The Use of Lactic Acid Bacteria for Vaccination

Inauguraldissertation
der Philosophisch-naturwissenschaftlichen Fakultät
der Universität Bern

vorgelegt von

Lorenz Scheppler

von Bubikon, ZH

Leiter der Arbeit:

Prof. Dr. Beda M. Stadler
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Der Dekan:
Prof. Dr. G. Jäger

Dominique

Ab imo pectore

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A. SUMMARY

The human mucosal surface is a site at which the host encounters a large variety of environmental microorganisms and through which human pathogens can initiate infections. However diverse mechanisms of defense ensure a permanent and efficient surveillance. A very important mechanism of protection at mucosal surfaces is the secretory mucosal immune system by producing specific secretory IgA against luminal antigens such as toxins, pathogenic bacteria and viruses to prevent their interactions with the epithelial surface. In addition to host derived defense mechanisms the gut microflora can play a major protective role by creating a "barrier effect" against pathogens. In recent years the food manufacturing sector has developed the so called functional food containing ingredients for promoting health. Probiotics represent a microbial food ingredient which beneficially is believed to affect the host by improving the properties of the indigenous microflora. Because of their proven safety lactic acid bacteria (LAB) are the most widely used group of probiotics.

The aim of the first part of this dissertation was to show that recombinant LAB can be used for mucosal, and especially oral immunisation purposes. Mucosal presentation of vaccines may offer a number of advantages over other routes: It can be carried out in a large scale and is relatively inexpensive. In addition mucosal immunisation induces both local and systemic immune responses. To generate a mucosal vaccine based on recombinant LAB, two Gram-positive genera of LAB, *Lactobacillus johnsonii* (Lj) and *Lactococcus lactis* (LcL) were used. As a model antigen, a mimotope derived from Tetanus toxin (TTmim) was fused to the C-terminal membrane anchoring domain of the *Lactobacillus bulgaricus* surface proteinase B. The TTmim was observed on the surface of the LAB. To determine whether recombinant LAB strains could induce an immune response, mice were immunised by oral administration of live recombinant bacteria. Results have shown that serum IgG and fecal IgA antibodies specific for PrtB can be detected following oral administration of recombinant Lj and to a lesser extend LcL bacteria indicating that LAB strains carrying a surface displayed antigen are suitable for oral immunisation purposes. However no antibody response was found against the TTmim, suggesting that the TTmim was too small for oral vaccination purpose (Dissertation Equivalent A).

In the second part of the dissertation we intended to use recombinant Lj as an active, mucosal anti-IgE vaccine. Previous reports have described the use of mimotopes of human IgE, Cε4 mimotopes (Cε4mim) or anti-idiotypic Fab (Fab α-Id-B43) that mimic IgE epitopes, to induce an anti-IgE response which has been shown to prevent allergic reactions. To generate a mucosal anti-IgE vaccine, recombinant strains of Lj were produced, by fusing the C-terminal 1615 amino acids of PrtB to either the Cε4mim or to an anti-idiotypic single chain fragment variable (α-IdscFv2) derived from Fab α-Id-B43. Both recombinant Lj strains were shown to express the heterologous fusion proteins and were recognised specifically by the protective anti-human IgE mAb BSW17. Subcutaneous and intranasal immunisation of mice with these recombinant Lj induced a systemic IgG response against human IgE. Thus our data suggest that recombinant Lactobacilli expressing IgE epitopes might be used for vaccination to induce in vivo a beneficial anti-IgE response as a novel immunotherapy (Dissertation Equivalent B).

B. SCIENTIFIC OVERVIEW

The mucosal Immune system

The human intestinal mucosal surface provides approximately 400 m^2 of available surface area for contact with antigens. In this part of the body mucosal immune responses form the first line of defence against numerous bacterial and viral diseases (1). Protection against infective agents is complemented intrinsically by the barriers of the gut-associated lymphoid tissues (GALT) (2). An intriguing aspect of the intestinal immune system is its ability to mount an energetic response against pathogens but not against commensal bacteria and harmless food antigens (3). For our health it is of major importance that the mucosal immune system can discriminate between situations where a protective immune response is absolutely required and where there should be no response. In order to explain this phenomenon it has been postulated that the default mechanism in the gut to nominal antigen is one of tolerance (4). But until now it has never been observed, that dietary feeding of antigen can completely eliminate immune responses against the same antigen following subcutaneous vaccination (5). The mucosal immune system has several unique properties in comparison to the blood borne immune system. One striking difference is the triggering of a systemic immune response after induction of a mucosal immune response (Table 1 and (6)). Another qualitative difference is the class of antibodies produced at mucosal sites that belong mainly to the IgA isotype in contrast to the blood borne immune system where IgG antibodies predominate.

Table 1
Immune response to antigens depends on the route of immunisation

Immune response / Immunisation	systemic	mucosal
systemic	+	-
mucosal	+	+

Cellular components of the mucosal immune system

A single layer of epithelial cells lines the entire intestinal mucosal surface. These cells form a barrier between the internal and external environment (Figure 1). Besides this function they are very important in the bidirectional signalling between the intestinal lumen and the mucosal immune cells. Behind and within the epithelial cells there is a special population of immune cells known as intraepithelial Lymphocytes (IEL). They belong mostly to the T cell lineage and most of them are CD8+ T cells expressing the αβ T cell receptor. Interestingly in most species including humans 10% of the IEL are γδ positive T cells. Located beneath the epithelial layer, in the so called Lamina propria, are mucosal lymphoid follicles; the Peyer's patches (PP). The apical part of the PP is built of differentiated microvilli cells, the M (membranous) cells. They serve to capture antigens from the intestinal lumen and to transport them to the underlying macrophages and lymphocytes (5, 7). The close proximity of antigen presenting cells (APC) and T cells in PP supports the idea that they are the primary sites for induction of mucosal immune responses. The T cells present in the parafollicular regions of the PP are mature and >95% express the αβ T cell receptor. From these 50-60% are CD4+ T helper cells (6). The main antigen-presenting cells in the PP are the dendritic cells. Like in all other lymphoid follicles the central regions of the mucosal lymphoid follicles bear the B-cell rich areas.

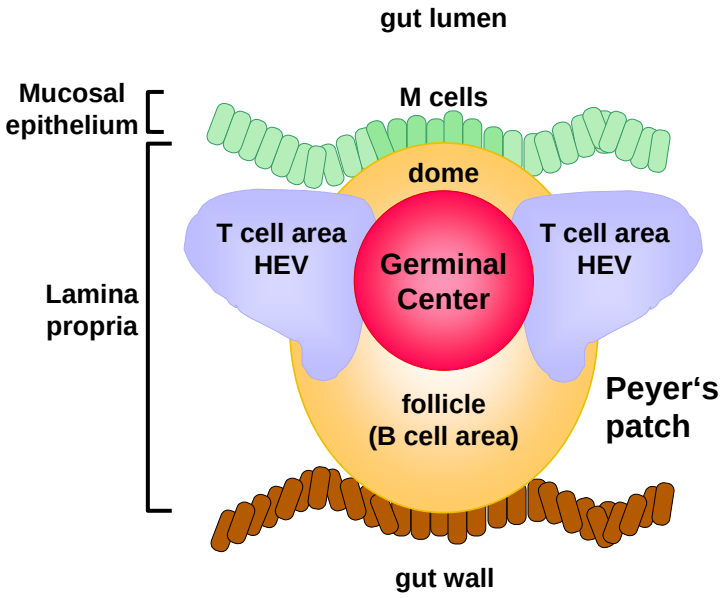

Figure 1: The cellular components of the mucosal immune system (schematic view)

Mucosal immune reaction

Orally administered antigens elicit a systemic response in the spleen after 2 to 7 days. Only after 7 to 10 days antigen-specific B cells are detected in the mucosal immune system. Mucosal immune responses are induced in the PP (Figure 2). The antigen is transported via the M cells to the basolateral surface of the PP, where it interacts with lymphoreticular cells to evoke an immune response. After the induction of the immune response activated T- and precursor B cells, that are committed to IgA synthesis under the influence of TGF-β and Il-5, leave the PP via efferent lymphatics. In the mesenteric lymph nodes they further mature and enter the systemic circulation through the thoracic duct. Via specific homing they ultimately enter the mucosal effector sites, such as the lamina propria of the respiratory, gastrointestinal and reproductive tracts as well as glandular tissues. This specific homing is mediated by the upregulation of α4β7 integrin on activated T and B cells. The interaction of these integrins with the mucosal vascular addressin (MAdCAM-1) leads to direct lymphocyte traffic into PP and the intestinal lamina propria. The consequence of this homing is the fact that up to 80% of all IgA producing plasma cells reside in the intestinal lamina propria (8). There the terminal differentiation into IgA plasma cells occurs under the influence of locally produced cytokines such as TGF-β, Il-5 and Il-6 (6, 9). Besides the specific secretory immunity the mucosal surfaces are protected by luminal secretions including mucus, glycolipids, protective peptides and antibiotic-like substances such as defensins.

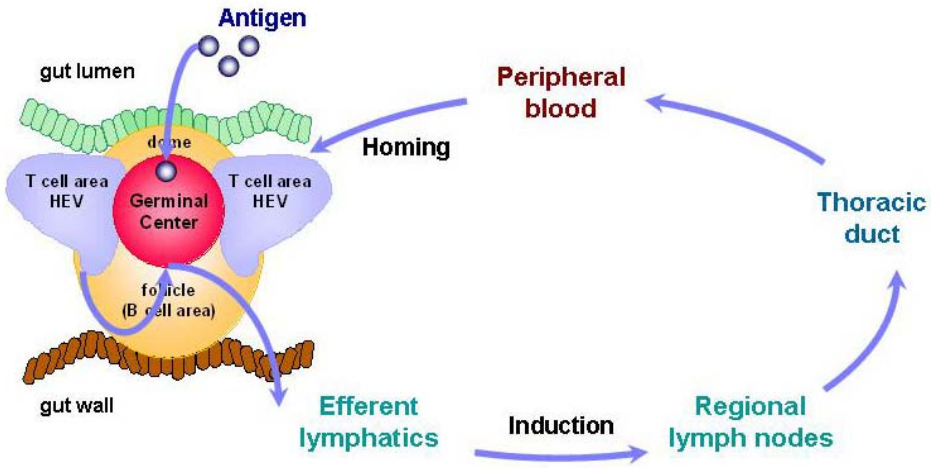

Figure 2: Schematic view of a mucosal immune reaction

The production of IgA

The hallmark of mucosal immune responses is the production of large amounts of antigen-specific secretory-IgA antibodies (s-IgA) (10). A normal 70 kg adult secretes about 3g IgA per day, about 60 to 70 percent of the total production of antibodies. Secretory IgA forms dimers that are held together by a coordinately synthesised and secreted J chain. From the Lamina propria the antibody-dimers are transported through the epithelial cell layer by a protein called the secretory component or poly-Ig receptor (Figure 3). After binding to the secretory component, the whole complex is endocytosed into the epithelial cell and transported to the luminal surface. Here the secretory component is proteolytically cleaved, and the extracellular domain with the attached IgA-dimers is released into the intestinal lumen. The secretory component itself is not only responsible for the transport of the IgA-dimers but also for stabilisation of the IgA in the harsh conditions of the intestine (6, 7). The s-IgA recognises and binds specific antigens and immune complexes are formed thus preventing antigens from interacting with the mucosal surface. This non-inflammatory mechanism of defence is known as immune exclusion, which is very efficient against pathogens (3).

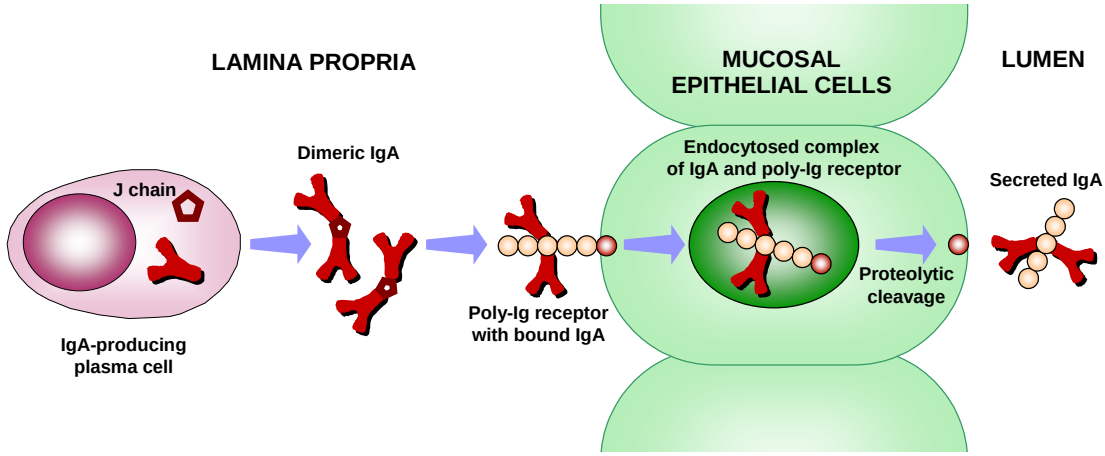

Figure 3: Transport of IgA through epithelial cells (derived from Abbas et al (7))

Atopic diseases

Epidemiology

During the last 30 years the prevalence of atopic diseases - asthma, rhinitis, eczema and food allergies – has considerably increased and it is estimated, that at least 20% of the population worldwide is suffering from an atopic disease (11). In Sweden the number of children with allergic rhinitis, asthma or eczema doubled during the years 1979-1991 (12). In Britain and Australia a recent report estimated 1 in 4 children under the age of 14 to have asthma and 1 in 5 to suffer from eczema (13). This striking increase cannot be simply attributed to improved diagnostic techniques nor to the increased awareness of the general public. The high morbidity associated with atopy leads to the requirement for large quantities of costly medication which relieve only the symptoms and have no effect on the underlying immune disorder. For example the annual costs of treating asthma in the United States were estimated at $6 billion in 1997 (14). Due to the large numbers of people affected much effort was laid into the investigation of the mechanisms and the development of novel therapeutic strategies. Strategies currently discussed include suppression, neutralisation and blocking not only the mediators of atopic disease but also the immunological mechanisms of atopy (15-17). Atopic allergic diseases are familial and genetic linkage studies have consistently identified a cytokine cluster on chromosome 5 and the MHC on chromosome 6 (18). However these genetic factors do not explain the rising incidence of atopic disease. Over the years there have also been many epidemiological studies on different population groups worldwide attempting to find relations between cause and effect. These studies are based on the idea that some environmental changes might have an influence. Although its clinical significance remains still unclear, events during the gestational period might play a role in determining whether a genetic predisposition becomes reality. However, after birth many external influences would also have to be considered in the search for cause and effect phenomena. These influences may be grouped under the term lifestyles which includes diet (19, 20), the so-called hygiene hypothesis (20-22) and its possible effect on the immune response and of course actual exposure to allergens.

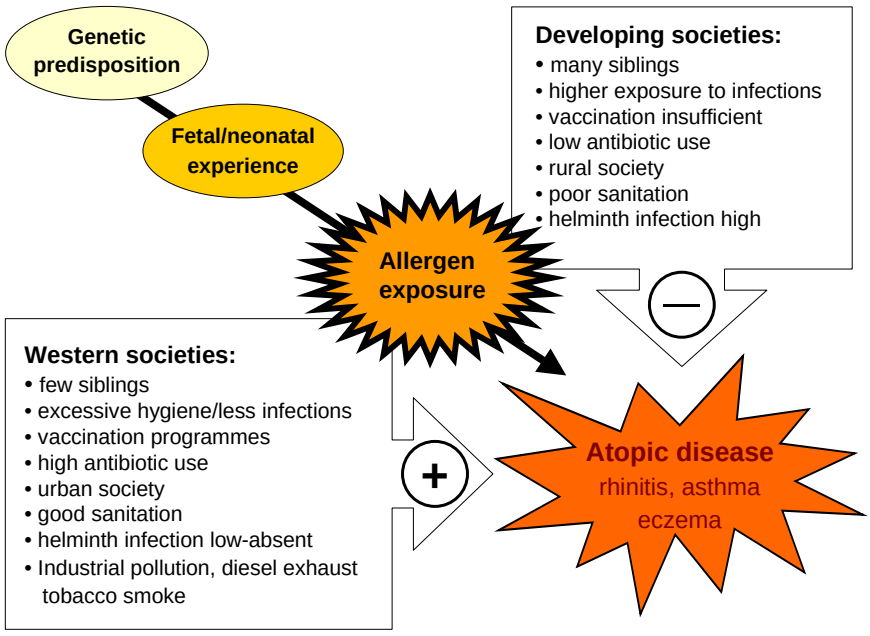

Figure 4: Multi-factorial determinants influencing the development of atopic disease (adapted from 23)

Allergens

It is not really known why some antigens cause allergic responses whereas others do not. The allergenicity may eventually reside in the biochemical properties of the antigens themselves. It is claimed that such properties include low molecular weight, glycosilation and high solubility in body fluids (23). The allergens that most commonly produce reactions in susceptible individuals are known as major allergens. Major allergens from the house dust mite *Dermatophagoides pteronyssinus* are Der p I and Der p II. Other important allergens are Fel d I from the cat *Felis domesticus*, several tree allergens including Bet v I from pollen of the birch tree (*Betula verrucosa*); and many grasses such as Phl p I and Phl p 5 from timothy grass (*Phleum pratense*) (24). Major allergens have also been associated to many different types of food including peanut, soybean, milk and shellfish (25). Particular allergens are associated with different types of allergic diseases. Grass pollens cause hay fever whereas allergens derived from house dust mites, cats and cockroaches induce asthma (26). The difference might be due to the size of the particles as pollen particles are too large to penetrate low into the respiratory tract. Allergies to food induce immediate hypersensitivity reactions that lead to release of mediators from intestinal mucosal cells and may represent a significant risk of fatal anaphylaxis (27). Interestingly allergenicity of food allergens seems to be associated with their stability in the gastrointestinal tract (28). Novel strategies using bioinformatics tools will probably give new insights for the further characterisation of allergens (29).

Initiation of Immediate Hypersensitivity

The defining hallmark of immediate hypersensitivity (type I hypersensitivity) is the production of specific IgE to some environmental protein antigens, commonly called allergens. The typical sequence of events in IgE-mediated immediate hypersensitivity reaction consists of multiple steps, beginning with initial exposure to an antigen (Figure 5).

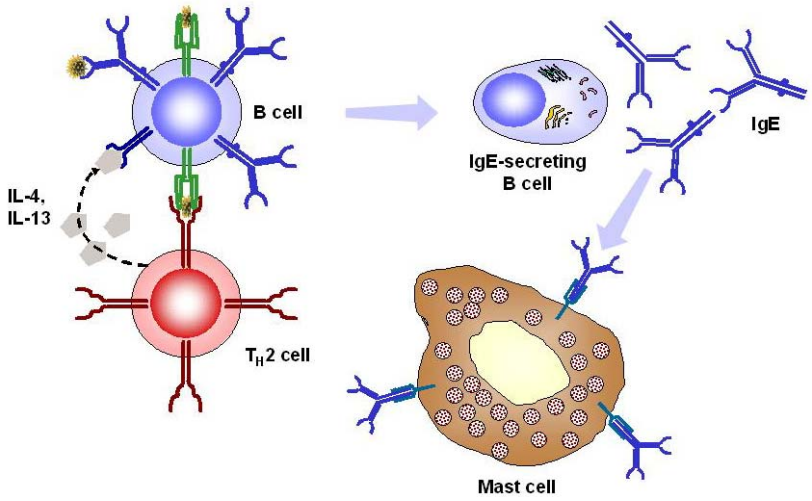

Figure 5: Induction of immediate hypersensitivity reactions: IgE production and binding to mast cells (adapted from 7)

In generally susceptible individuals, CD4+ Helper T differentiate into T_H2 effector cells (30). T_H2 cells play a central role in the regulation of the IgE antibodies by producing Il-4 and Il-13 (31, 32). These are the only known cytokines that are able to induce IgE synthesis in vitro when added in recombinant form (31). In addition to the production of cytokines T_H2 cells

also provide contact-mediated signals that B cells require to undergo IgE isotype switching. These contact-mediated signals include the interaction of the antigen-specific T cell Receptor (TCR) to the MHC II associated and processed allergenic peptide on the B cell, the subsequent interaction between induced CD40L on the T cell and constitutively presented CD40 on the B cell and the binding of induced CD80 (B7-1) and CD86 (B7-2) to CD28 (33). It is the interaction of CD40 and its ligand that promotes IgE class switching, B-cell growth and other functions (34). The produced IgE circulates throughout the body and binds to the high affinity Fc receptors specific for the ε chain on the surfaces of circulating basophils and on mast cells in various tissues (35). If the antigen is reintroduced, it binds to the cell-associated IgE and can cross-link two or more IgE molecules and the Fc receptors to which they are bound (Figure 6). Cross-linking of the Fc receptors initiates signal transduction events in the mast cells and basophils that result in the release of preformed mediators stored in the cytoplasmic granules of these cells and the rapid synthesis and release of lipid mediators and cytokines (Figure 6). Some of these mediators induce an early (immediate) vascular and smooth muscle reaction, and others stimulate the influx of leukocytes resulting in the late phase reaction (reviewed in 36-38).

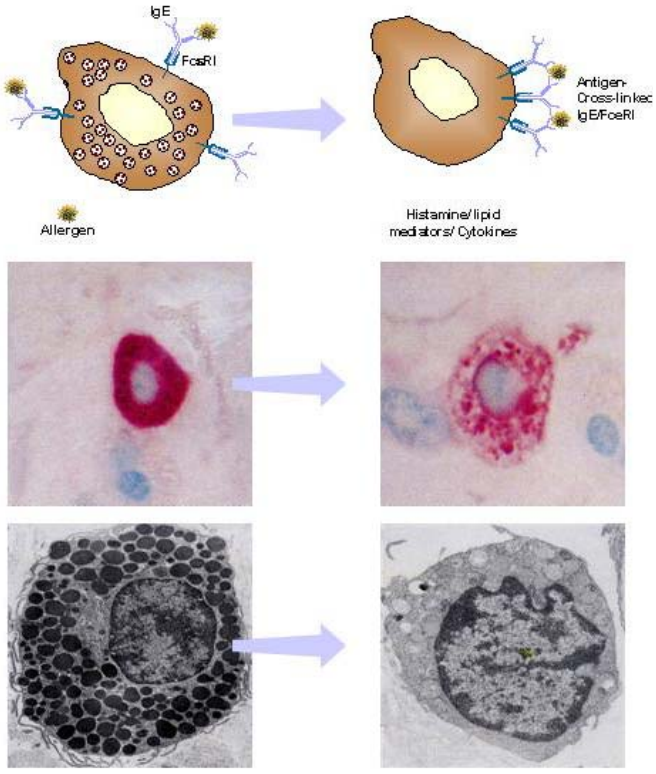

Figure 6: Mast cell activation (adapted from 7)

The FcεRI-IgE interaction

The biologic effects of IgE in immediate hypersensitivity are mediated by a high-affinity Fc receptor specific for ε heavy chains, called FcεRI, that is constitutively expressed on mast cells and basophils and on many other cells of the immune system as eosinophils, monocytes, macrophages, Langerhans cells, platelets, megakaryocytes and recently neutrophils. IgE functions as an antigen receptor on the surface of mast cells and basophils. This function is accomplished by IgE binding to FcεRI on these cells. The dissociation constant (K_d) is about 1×10^{-10} M, which is stronger than that of any other Fc receptor for its ligand (38). Each

FcεRI molecule is composed of three separate transmembrane subunits (Figure 7), one α chain that mediates ligand binding, and three chains that contribute to signalling, including one β chain and a dimer of identical γ chains (39). Both the α and γ chains must be present to have cell surface expression (35). In macrophages, monocytes, Langerhans cells and dendritic cells the β chain is lacking (αγ2), but on IgE dependent effector cells, mast cells and basophils, the receptor is composed of αβγ2. The amino terminal extracellular portion of the α chain includes two Ig-like domains that form the binding site for IgE.

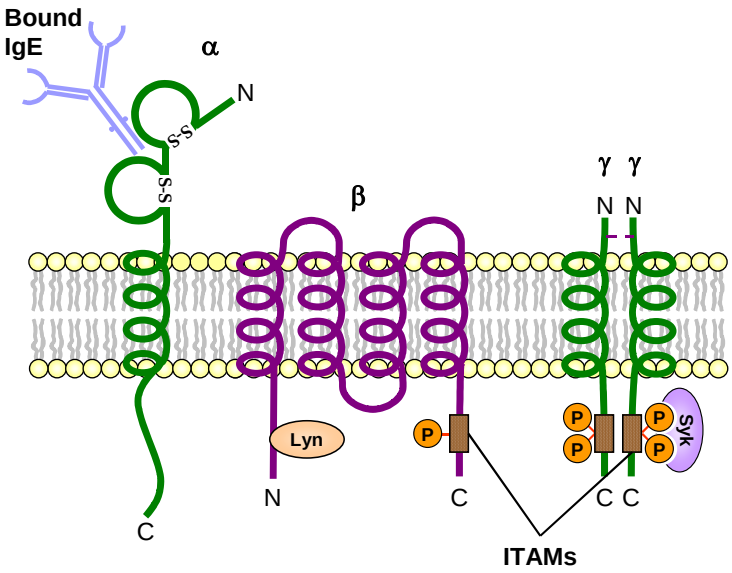

Figure 7: Polypeptide chain structure of the high-affinity IgE Fc Receptor (FcεRI, adapted from 7)

The β chain of FcεRI, which crosses the membrane four times, contains a single immunoreceptor tyrosine-based activation motif (ITAM) in the carboxy terminus. Together with the two ITAM of the two γ chains, which form a disulfide-linked homodimer, the ITAM are responsible for the signalling functions of the FcεRI.
The structure of IgE consists of two heavy chains, each of 1 variable and 4 constant domains (Cε1-4), that associate either with κ or λ-light chains. The half-life of IgE in solution is approximately 2.5 days and increases when IgE is complexed with IgG in small immune complexes. IgE has a bent structure (40) and binds via the convex side of the molecule with its Cε3-domains to the FcεRI. Due to steric hindrance only one FcεRI can bind to an IgE molecule. The interaction between the receptor and IgE stabilises the complex and the half-life of the IgE- FcεRI complex is approximately 21 days.

Effector Functions of Mast cells and Basophils

The effector functions of mast cells and basophils are mediated by soluble molecules released from the cells upon activation (Table 2). These mediators may be divided into preformed mediators which include biogenic amines and granule macromolecules, and newly synthesised mediators, which include lipid-derived mediators and cytokines.
Many of the biologic effects of mast cell activation are mediated by biogenic amines that are stored in and released from cytoplasmic granules (reviewed in 41). Biogenic amines, sometimes called vasoactive amines, are nonlipid low molecular weight compounds that share the structural feature of an amine group. In human mast cells, the only mediator of this class

that is present in significant quantities is histamine, but in certain rodents serotonin may be of equal or greater import. Histamine acts by binding to target cell receptors.

Table 2
Mediators produced by Mast cells, Basophils and Eosinophils (41)

Mediator category	Mediator	Function/ Pathologic Effects
Mast cell		
Preformed, stored in cytoplasmic granules	Histamine	Increases vascular permeability; stimulates smooth muscle cell contraction
	Enzymes: neutral proteases	Degrade microbial structures; tissue damage/remodelling
Major lipid mediators produced on activation	Prostaglandin D_2	Vasodilation, bronchoconstriction, neutrophil chemotaxis
	Leukotriene C_4, D_4, E_4	Prolonged bronchoconstriction; mucus secretion, increased vascular permeability
	Platelet-activating factor	Chemotaxis and activation of leukocytes, bronchoconstriction, increased vascular permeability
Cytokines produced on activation	IL-3	Promotes mast cell proliferation
	TNF-α, MIP-1α	Promotes inflammation/late phase reaction
	IL-4, IL-13	Promote T_H2 differentiation
	IL-5	Promotes eosinophil production and activation
Basophils		
Preformed, stored in cytoplasmic granules	Histamine	Increases vascular permeability; stimulates smooth muscle cell contraction
	Enzymes: neutral proteases	Degrade microbial structures; tissue damage/remodelling
Major lipid mediators produced on activation	Leukotriene C_4	Prolonged bronchoconstriction; mucus secretion, increased vascular permeability
Cytokines produced on activation	IL-4, IL-13	Promote T_H2 differentiation
Eosinophils		
Preformed, stored in cytoplasmic granules	Major basic protein Eosinophil cationic protein	Toxic to helminths, bacteria, host cells
	Eosinophil peroxidase, Lysosomal hydrolases, Lysophospholipase	Degrades helminthic and protozoan cell walls; tissue damage/remodelling
Major lipid mediators produced on activation	Leukotriene C_4, D_4, E_4	Prolonged bronchoconstriction; mucus secretion, increased vascular permeability
	Lipoxins	Promote inflammation
Cytokines produced on activation	IL-3, IL-5, GM-CSF	Promote eosinophil production and activation
	IL-8, IL-10, RANTES, MIP-1α, eotaxin	Chemotaxis of leukocytes

The action of histamines are short lived because histamine is rapidly removed from the extracellular milieu by amine-specific transport systems. Binding of histamine to endothelium causes cell contraction leading to leakage of plasma into the tissues (Figure 8). Histamine also stimulates endothelial cells to synthesise vascular smooth muscle relaxants, such as prostacyclin (PGI_2) and nitric oxide, which cause vasodilation. These actions of histamine produce the wheal and flare response of immediate hypersensitivity. Histamine also causes constriction of intestinal and bronchial smooth muscles. Thus histamine may contribute to the increased peristalsis and bronchospasm associated with ingested allergens and asthma.

Neutral serine proteases, including tryptase and chymase, are the most abundant protein constituents of mast cell secretory granules. The presence of tryptase in human biologic fluids is interpreted as a marker of mast cell activation. Their biological function *in vivo* still remains unclear.
Mast cell or basophil activation also results in the rapid de novo synthesis and release of lipid-derived mediators that have a variety of effects on blood vessels, bronchial smooth muscle and leukocytes. The most important of these mediators are cyclooxigenase and lipooxygenase metabolites of arachidonic acid. The major mediator from this class produced in mast cells is prostaglandin D_2 (PGD_2), whereas Basophils do not produce significant quantities. PGD_2 acts on smooth muscle cells and as a vasodilator and bronchoconstrictor. It also promotes neutrophil chemotaxis and accumulation at inflammatory sites. The most important lipid mediator in mast cells and basophils are the leukotrienes, especially leukotriene C_4 (LTC_4).

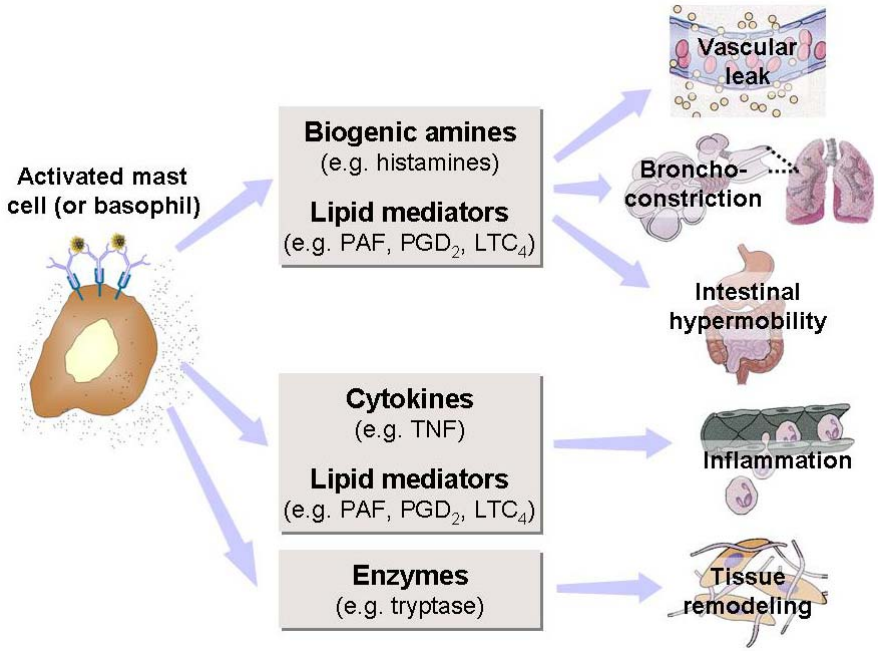

Figure 8: Biologic effects of mediators of immediate hypersensitivity (adapted from (7))

LTC_4 causes prolonged bronchoconstriction and is an important factor for asthmatic bronchoconstriction. A third type of lipid mediator produced by mast cells is called platelet-activating factor (PAF). PAF also shows direct bronchoconstricting actions.
Mast cells and basophils also produce many different cytokines that may contribute to allergic inflammation. These cytokines include TNF-α, IL-1, IL-4, IL-5, IL-6, IL-13, MIP-1α, MIP-1β and various colony-stimulating factors such as IL-3 or GM-CSF. It seems likely, that cytokines released upon IgE-mediated mast cell or basophil activation are predominantly responsible for the late phase reaction.

Allergic Diseases in Humans

Mast cell degranulation is a central component of all allergic diseases, and the pathologic manifestations of the diseases depend on the tissues in which the mast cell mediators have effects, as well as the chronicity of the resulting inflammatory process. The most common forms of atopic disease are allergic rhinitis (hay fever), bronchial asthma, atopic dermatitis (eczema) and food allergies. Usually the point of contact with the allergens determines the organs or tissues that are involved. The concentrations of mast cells in various target organs influence the severity of responses. Mast cells are particularly abundant in the skin and the

mucosa of the respiratory and gastrointestinal tracts, and these tissues frequently suffer the most injury in immediate hypersensitivity reactions.
In systemic immediate hypersensitivity, mast cells and basophil mediators gain access to vascular beds throughout the body and cause vasodilation and exudation of plasma. The decrease in vascular tone and leakage of plasma can lead to a fall in blood pressure or shock called anaphylactic shock, which is often fatal. Bronchial asthma is an inflammatory disease caused by repeated immediate hypersensitivity reactions in the lung leading to the clinicopathologic triad of intermittent and reversible airway obstruction, chronic bronchial inflammation with eosinophils, and bronchial smooth muscle cell hypertrophy and hyperreactivity to bronchoconstrictors. Although most cases of asthma are due to immediate hypersensitivity, in about 30% of the patients asthma may not be associated with atopy and may be triggered by nonimmune stimuli such as drugs, cold and exercise. Allergic rhinitis is perhaps the most common allergic disease and is a consequence of immediate hypersensitivity reactions to common allergens such as plant pollen or house dust mites localised to the upper respiratory tract by inhalation. The pathological and clinical manifestations include mucosal edema, leukocyte infiltration with abundant eosinophils, mucus secretion, coughing, sneezing and difficult breathing. Allergic conjunctivitis with itchy eyes is commonly associated with the rhinitis. Food allergies are immediate hypersensitivity reactions to ingested foods that lead to the release of mediators from intestinal mucosal and submucosal mast cells. Clinical manifestations include enhanced peristalsis, increased fluid secretion from intestinal lining cells and associated vomiting and diarrhoea. Manifestation of allergic reactions in the skin are the wheal and flare reaction, a reflection of the acute effects of mast cell mediators, followed by swelling and induration, a reflection of the inflammatory events of the late phase reaction. These skin manifestations can occur in response to direct contact with the allergen or after an allergen enters the circulation via the intestinal tract or by injection. Skin reactions are largely mediated by histamine.

Therapy of allergic disorders

Current clinical concepts

Current therapeutic approaches aimed at the treatment of allergic disorders include allergen avoidance, anti-allergic medication and immunotherapy for specific allergens, also known as hyposensitisation or desensitisation. The most frequently used drugs currently used are histamine antagonists and anti-cholinergic agents for the relief of symptoms and corticosteroids for the suppression of allergic inflammation. Recent advances in the understanding of the inflammatory and immunological processes of atopy have led to the development of new therapeutic strategies for allergic diseases (42, 43). Promising approaches such as anti-IgE immunotherapy and, DNA vaccination, CpG oligonucleotides and mycobacterial vaccination are of considerable interest to prevent or cure atopic diseases in the future (reviewed in 23).

Specific allergen Immunotherapy, Modulation by Cytokines and Vaccination

Conventional allergy immunotherapy consists of administering small but increasing amounts of allergens over a long period of time. It has been successfully used for the treatment of allergic rhinitis (44), allergic asthma and insect venom anaphylaxis (45), and can induce prolonged remission. It is believed, that immunotherapy shifts the immune response from the production of IgE to the production of neutralising IgG antibodies (46). Several studies have shown that immunotherapy inhibits the release of mediators, prevent the recruitment of eosinophils into the skin and nose, decreases the number of mast cells in subcutaneous tissues and may also shift the balance from a T_H1 to a T_H2-type cytokine production (47). Although

immunotherapy is successful in reducing the clinical symptoms in 60-90% of the cases few patients are cured (48). The occurrence of anaphylactic reactions and even death in a few patients has led to the development of other strategies using recombinant allergens that have reduced allergenic and anaphylactic activity. Other strategies involve the use of drugs that inhibit cytokine synthesis such as glucocorticoids, cyclosporine A, cytokine antagonists such as antibodies, soluble receptors (49), or cytokine mutants, as well as drugs that block the signal-transduction pathway activated by cytokines. Furthermore allergen T-cell peptides or epitopes have been used instead of whole allergens, which prevents the cross-linking of allergen specific IgE on mast cells and basophils. In mouse models, treatment with peptide derived from Fel d I, can prevent immediate hypersensitivity and airway hyperresponsiveness (50). However side effects were reported with all peptide strategies used. According to the theory that the lack of microbial stimulations in early life might play a role in the development of atopy in genetically pre-disposed individuals, early infection with viruses or bacteria might trigger a T_H1 immune response which subsequently may inhibit T_H2 immune responses and protect against both infections and allergic reactions (51). Immunisation using Bacille Calmette-Guérin (BCG) however showed no clear conclusion as to whether it can inhibit atopy in humans. Other approaches using DNA vaccination strategies proved to be more promising when DNA encoding for Der p 5 was used in a rat model (52). Yet the efficacy of DNA vaccines in patients with atopic diseases has to be evaluated. In animal models also CpG (cytosine-phosphate-guanosine) oligodeoxynucleotides (CpG-ODN) proved to be functional (53) but also in this case the question remains, whether CpG can be used for the treatment of patients.

Passive anti-IgE therapy

One of the most promising therapeutic approaches is the neutralisation of IgE by antibodies that are directed against the region of IgE which is involved in the binding of IgE to the receptors. Monoclonal anti-IgE antibodies to mouse IgE were isolated that did not induce histamine release as they react with an IgE epitope recognised by FcεRI (54). Later, murine monoclonal anti-IgE antibodies to human IgE were also generated that bound to IgE but were non-anaphylactogenic and inhibited the binding of IgE to FcεRI making them attractive candidates for therapeutic application (55, 56). The target structure of these antibodies has been located to the third constant domain of IgE (57), which is consistent with the binding site of the FcεR. All antibodies have in common, that they interfere with the binding of IgE to its Fc receptors. One of these antibodies, termed BSW17 (Figure 9), was shown to recognise receptor-bound IgE without triggering human basophils (58), probably by inducing a conformational change of the IgE molecule. This antibody was also shown to inhibit IgE synthesis upon bivalent recognition of surface IgE (59).

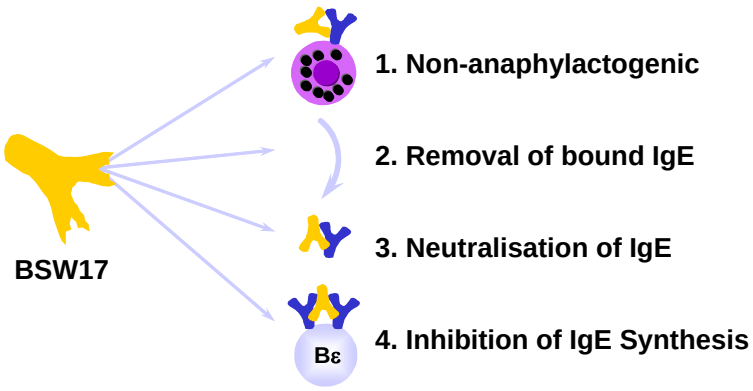

Figure 9: Properties of the murine anti-human IgE mAb BSW17

To avoid the problem of antigenicity in humans until now two murine anti-human IgE antibodies have been humanised using a method developed by Riechman et al (60). Amongst several other variants rhuMAb-E25 was chosen for further clinical development. Toxicity studies have demonstrated no evidence of serum sickness, hypersensitivity reaction or the development of anti-E25 antibodies. Administration of E25 induces a dose-dependent decrease of serum free IgE level to 1% of pre-treatment level associated with a marked down-regulation in basophil FcεRI expression and histamine release (61). Results from clinical trials clearly showed an improvement in symptoms and reduced use of medication in moderate to severe asthma (62). This treatment should be available in the near future and would be used initially for patients with severe atopic asthma whereas more results are required to evaluate the benefits of E25 in non-atopic asthma and mild asthma.

Active anti-IgE treatment

An alternative strategy for neutralising IgE is active immunisation using IgE epitopes in order to induce a natural autoimmune response, resulting in the formation of autoantibodies that have the same effect as passively administered non-anaphylactogenic anti-IgE antibodies. To isolate such IgE epitopes BSW17 was used as the selecting antigen (63, 64). By screening random peptide phage display libraries two peptides were isolated that mimic the epitope recognised by BSW17 on the IgE molecule. Therefore the peptides were called mimotopes. One of this mimotopes, Cε4 mimotope, showed a structural homology to a part of the Cε4-domain of human IgE (64), whereas the Cε3 mimotope exhibited sequence homology within an epitope located in the Cε3 domain of IgE (65). As both mimotopes were exclusively recognised by BSW17 and were able to inhibit binding of BSW17 to IgE BSW17 probably has two binding sites on the IgE molecule. Coupled to keyhole limpet hemocyanin (KLH) the mimotopes were then used to immunise Rhesus monkeys. In an in vivo passive cutaneous anaphylaxis (PCA) test both mimotopes were shown to be functional. In addition the Cε4 mimotope was shown to induce reduced PCA activity even after 10 months. The immune reaction could be boosted by reapplying Cε4 mimotope, indicating that active immunisation was able to induce memory B cells. According to Jerne's network hypothesis, the binding site of an anti-idiotypic antibody also represents the internal image of an epitope present on a foreign, or even a self-antigen (66). Therefore it should be feasible to find anti-idiotypic antibodies with structural homology to isolated random peptides, mimotopes of IgE (Figure 10). To isolate such antibodies, a pool of human Fab phage display libraries was used that had been isolated from peripheral blood mononuclear cells of allergic and non-allergic donors and from purified B cells from children's tonsils.

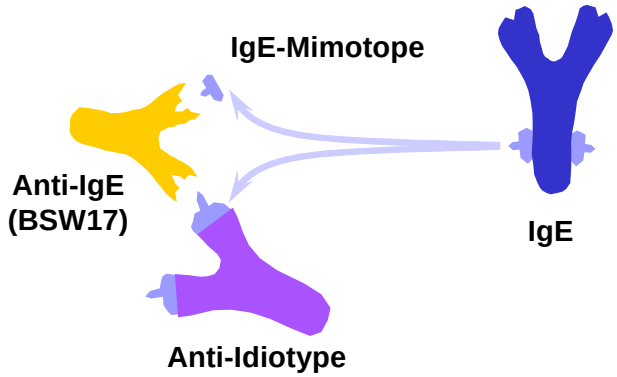

Figure 10: Mimicry of IgE epitope by either IgE mimotope or anti-idiotypic antibody (67).

Upon selection on BSW17 two anti-idiotypic antibodies were isolated, that exclusively interacted with BSW17. Interestingly these antibodies mimic the same molecular region as the previously described IgE mimotopes, but they cover a much larger epitope on the IgE molecule (67). Again both anti-idiotypic antibodies were functional in a PCA test in rhesus monkeys. Experiments are continuing to evaluate whether these antibodies show the same long-term protection that was observed with the Cε mimotopes. In a vaccination procedure these anti-idiotypic antibodies may be better tolerated than peptides that have to be coupled to immunogenic carriers. Clinical trials will show whether the results obtained in monkeys are confirmed and whether an anti-IgE immune response will be induced that prevents binding of IgE to FcεRI and consequently the sensitisation of the effector cells involved in allergic disease.

Functional Food and Probiotics

In recent years, the food manufacturing sector has promulgated the importance of functional foods as dietary supplements for promoting health. A wide range of additionals such as vitamins, mineral salts, roughage and some probiotic strains of bacteria are used today. Probiotics represent a microbial food supplement that is said to beneficially influence the host by improving its intestinal balance. But altough much work has been done in this field until now there is no convincing proof, that animals fed with probiotics are resistant to experimental challenge with a pathogenic microorganism (5).

Commensal gut flora

There is general agreement on the importance of the gastro-intestinal (GI) microflora in the health status of men and animals. It is estimated that the colon of a healthy adult harbours about 400 different cultivable species, alltogether approximately 10^{15} bacteria (68). This microflora entertains a symbiotic interaction with its host. The 'normal' flora has many important functions. It does not only form a barrier against pathogenic and opportunistic microorganisms but it is also involved in the human metabolism, producing certain B vitamins and short chain fatty acids. But also minor groups of pathogenic and opportunistic organisms are always present in low numbers. Disturbance of the ecological balance in the Gastrointestinal tract (GIT) can be detrimental to health. The stable state of balance of the microbial population in the GIT is called 'eubiosis', the opposite situation is called 'dysbiois'. So it is not astonishing that an increased interest exists in possibilities of manipulating the composition of the gut microflora by food or food ingredients. The aim is to increase the numbers and activities of those microorganisms suggested to possess health promoting properties such as *Bifidobacterium* and *Lactobacillus* species (2). One good example, that the diet can influence the ratio between the microbial species and strains of the intestinal flora, is the colonisation of new-born children. During delivery the children's GIT is colonised with microorganisms from both the birth canal of the mother and from the environment. At first *Escherichia coli* and *Streptococcus* predominate, but in breast fed infants there is a sharp increase in the numbers of *Bifidobacterium* together with a concomitant decrease of *E. coli* and *Streptococcus*. In contrast formula fed babies do not show this shift, and their GI flora becomes rather complex with relatively high numbers of *Bacteroides, Clostridium* and *Streptococcus* (2).

Probiotic bacteria

During the last two to three decades attempts have been made to improve the health status by modulating the intestinal flora with live microbial adjuncts, now called probiotics. Probiotics are defined as "mono- or mixed cultures of live microorganisms which, when applied to animal and man, beneficially affect the host by improving the properties of the indigenous microflora" (69). Although probiotic microorganisms are considered to promote health, the actual mechanisms involved have not yet been fully elucidated. In addition to desirable technical features, like the possibility to cultivate the organisms on an industrial scale, factors related to health promotion or health sustaining serve as important criteria for strain selection. Many key criteria have been defined as desirable for probiotic bacteria (69). The key aspects refer to safety (non-pathogenic), survival of the defence system located in the upper regions of the human GIT (saliva, gastric and bile juice), presumable human origin and genetic stability.
Numerous beneficial effects have been suggested to result from probiotic activities in the gut. Probiotics modulate the immune system and strengthen the gut mucosal barrier due to the

modification of the gut microflora, the adherence to the intestinal mucosa with capacity to prevent pathogen adherence (70) or pathogen activation, the competition for metabolic substrates, the modification of dietary proteins by the intestinal microflora, the modification of bacterial enzyme capacity especially of those suggested to be related to tumour induction and the influence on gut mucosal permeability (2).

Functional foods using probiotic bacteria

Different product types or supplements containing viable microorganisms with probiotic properties are now commercially available either in a lyophilised form or as fermented food commodities. In present-day commercial products, Lactobacillus spp are well represented, followed by Bifidobacterium spp, some other Lactic acid bacteria (LAB) genera and even a few non-lactics (3). Table 3 summarises the microorganisms used for probiotic food.

Table 3
Microorganisms applied in probiotic products (2)

Lactobacillus species	***Bifidobacterium species***	***Other LAB***	***Non-lactics***
L.acidophilus	*B.adolescentis*	*Ent. faecalis* [a]	*Bacillus cereus* [a,d]
L.casei	*B.animalis*	*Ent. faecium*	*Escherichia coli* [d]
L.crispatus	*B.bifidum*	*Lactococcus lactis* [c]	*Propionibacterium freudenreichii* [a,d]
L.gallinarum [a]	*B.breve*	*Leuc.mesenteroides* [c]	*Saccharomyces cerevisiae* [d]
L.gasseri	*B.infantis*	*Ped.acidilactici* [c]	
L.johnsonii	*B.lactis* [b]	*Sporolactobacillus inulinus* [a]	
L.plantarum	*B.longum*	*Strep. Thermophilus*	
L.reuteri			
L.rhamnosus			

[a] Mainly used for animals
[b] Probably synonymous with *B.animalis*
[c] Little known about probiotic properties
[d] Mainly as pharmaceutical preparations

A steadily increasing range of yogurt-like products is available on the European market. Probiotic strains for these products are generally derived from the GIT of the adult human host (2). The underlying hypothesis of this type of functional food is to increase the beneficial components of the gastro-intestinal microflora. Two main strategies are used:

- Regular administration of an inoculum of live bacteria, which will interact with and improve the functions of the intestinal microflora - the probiotic concept (71).
- Administration of a bacterial substrate (i.e. oligosaccarides) that will promote the preferential growth of beneficial microorganisms such as bifidobacteria - the prebiotic concept (72).

Lactic acid bacteria

Lactic acid bacteria (LAB) represent a group of different gram-positive microorganisms. Since a long time LAB have been involved in food processing and food preservation. They are used in particular for the manufacturing of dairy products, fermented meat, vegetables, bread and ensilage. Many studies have shown that fermentation using LAB improves the nutritional value of food products by increasing the quantity, availability, digestibility and assimilability of nutrients. Yogurt for example contains higher levels of free amino acids as compared to milk due to proteolysis by the yogurt microflora. Research carried out in recent years has led to the conviction, that certain strains of LAB, especially strains from the genera *Lactobacillus* and *Bifidobacterium*, may promote health in man and animals. For example LAB are capable of synthesising certain vitamins (e.g. folic acid) (73). Oral or parenteral administration of LAB may even strengthen non-specific mechanisms of defense against infection and tumors. Some LAB were shown to inhibit *Helicobacter pylori* in vitro (74). Although not yet fully understood, the mechanisms involves the bactericidal effect of lactic acid, as well as the production of bacteriostatic compounds (75). As an interesting side effect LAB may act as adjuvants in antigen-specific immune responses (76).

Lactobacillus johnsonii

The genus *Lactobacillus* comprises more than 50 species widely differing in biochemical, ecological, molecular-biological and imunological properties (73). One of them, *Lactobacillus johnsonii* (Lj) is a gram-positive, rod-shaped, non-pathogenic microorganism (Figure 11; 77). It is a generally regarded as save (G.R.A.S.) organism. Lj like other members of the *Lactobacillus* genus is surrounded by a surface layer of proteins (S-layer). This S-layer is the result of the crystallisation of a single protein (S-protein). Although the functional significance of S-layers is not yet known, they can be expected to be important structural units in prokaryotes due to the substantial energy burden caused by their synthesis and export.

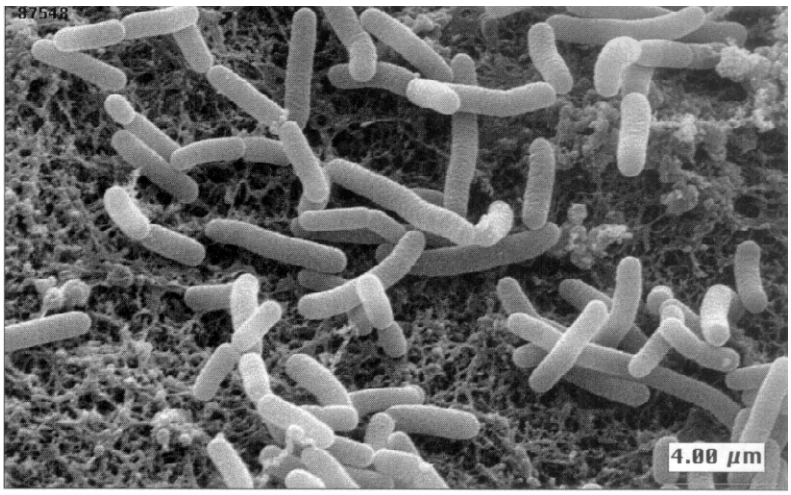

Figure 11: *Lactobacillus johnsonii*

Lactobacilli derive the energy necessary for growth largely from the fermentation of carbohydrates which leads to the production of lactic acid. They are widely known for the capacity to ferment food components, for supposed health promoting (probiotic) properties and as normal constituents of the human gut flora. It is believed that with the aid of *Lactobacillus* fermented food, protein malnutrition and vitamin deficiencies might be battled.

As with other LAB the oral administration of some *Lactobacillus* strains is positively correlated with reduction of serum cholesterol levels, detoxification of potential carcinogens, stimulation of host non-specific immunity (adjuvanticity) and inhibition of the outgrowth of microorganisms causing infectious diseases.
A particularly attractive feature of Lj, but not of *Lactococci*, is the resistance to gastric acidity and bile toxicity in the gastrointestinal tract. Surviving La 1 adhere to and maintain at mucosal tissues (78), thereby colonising the intestinal tract. Lj have been shown to adhere to human intestinal epithelial cells (Caco-2 cells) (79). This colonisation and the adhesion may play an important role in both specific and non-specific immune stimulation (73). The above findings and the property of Lj not to stimulate a strong immune response against itself makes *Lactobacillus* a very attractive candidate for use as an oral vaccine delivery system.

Lactococcus lactis

Lactococcus lactis (LcL; Figure 12) is a non-pathogenic, non-invasive and non-colonising gram-positive lactic acid bacterium (LAB). As Lj it is a generally regarded as safe (G.R.A.S.) organism. The bacterium is not adapted for growth in vivo and it does not belong to the commensal species of LAB. Under normal conditions LcL is destroyed during stomach passage (own results). However it is thought to be a good oral vaccine delivery system, because of the particulate form in which recombinant antigens are presented to the immune system. LcL is approximately the same size as biodegradable microparticles which are known to be readily taken up by M cells, and to be capable of acting as effective oral vaccine vehicles (80).

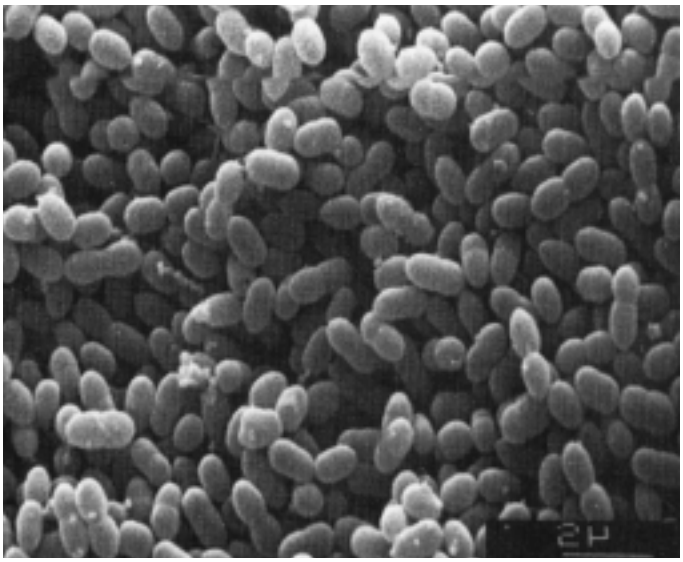

Figure 12: *Lactococcus lactis*

Oral immunisation and Vaccines

Vaccine developments

Vaccination as a means of preventing infectious disease has had the most successful impact on human health of any medical intervention. All vaccines intend to induce an immune response designed to prevent infection or limit the effects of infection (81). Another critical element of vaccines is memory. Because immunisation may take place many years before exposure to the pathogen, a long-lived immune response is called for.
During the last 200 years, vaccination has had a beneficial impact in the prevalence of major diseases such as small pox, tetanus, pertussis, diphtheria, poliomyelitis, measles, mumps, rubella and yellow fever (82). A wide range of different approaches have been taken to produce the variety of vaccines fulfilling these requirements.

The most obvious vaccine modality is to stimulate protection against a serious illness by prior infection with a weaker or related version or lower inoculum of a pathogen. The most famous example of this kind of vaccine is the immunisation used by Edward Jenner in 1796, who used harmless cowpox as a related immunogen against smallpox. The eradication of smallpox and widespread use of other live virus vaccines (e.g. tuberculosis) shows the great success of attenuated viruses. Efforts are now being made to use the tools of molecular biology to attenuate pathogens; specific mutations can be made by mutating or deleting a gene that encodes a protein responsible for a virulence factor.

Attenuated viruses and bacteria can be modified for use as carriers by inserting genes encoding a protein from a different pathogen into their genome. This vectors can be used for the delivery of genes from pathogens which themselves might be considered unsafe as an attenuated vaccine (e.g. HIV).

Replicons can be engineered to consist of a RNA-virus coat in which the structural genes have been replaced by the sequence encoding the antigen. After infection of a cell large quantities of mRNA and therefore large quantities of protein antigen are made, yet the virus (normally alpha viruses) cannot replicate because it no longer contains the sequences for the necessary structural proteins.

Subunit vaccines represent technologies ranging from the chemical purification of components of the pathogen grown in vitro (e.g. polysaccharide capsules of *S. pneumoniae*) to the use of recombinant DNA technology to produce a single viral protein (such as hepatitis B surface antigen).

It is a logical progression from whole inactivated viruses or bacteria to purified components of the pathogen; then to recombinant proteins and finally to peptide vaccines. In this approach, known B-cell or T-cell epitopes are the immunogen, generally coupled to a carrier molecule or organism designed to increase the immunogenicity of the epitope.

Today the greatest efforts are done in the field of nucleic acid vaccines which are typically bacterial plasmids carrying genes encoding pathogen or tumour antigens. The plasmids generally use a strong viral promoter to drive the expression of the gene of interest. In a rapidly increasing number of animal models, DNA vaccines have been shown to be effective at generating protective immune responses against a wide variety of diseases (83).

Oral immunisation

Oral presentation (immunisation) of antigens offers a variety of advantages over parenterally administered vaccines. Oral administration of vaccines is convenient, can be carried out on a large scale and is relatively inexpensive. In addition, oral immunisation may yield, for a number of pathogens, herd immunity, which means that immunity is spread among the members of the community following immunisation of only a small proportion of individuals (84). These advantages are particularly relevant when vaccines are to be applied in less industrialised countries. Furthermore, many bacterial, viral and parasitic pathogens enter the body via the mucosal surfaces of the body where more than 60% of antibodies are secreted daily. Finally, oral immunisation frequently evokes both local and systemic immune responses, resulting in an effective elimination of foreign invaders (73). But until today only one orally administered vaccine is available, the vaccination against Polio using a formalin-inactivated form of the virus.

Recognition of the significance of the mucosal immune system has resulted in the development of experimental mucosal antigen delivery systems. Because the vaccine has to survive the harsh conditions of the stomach and the upper intestinal tract, the vaccine itself has to be protected. For experimental approaches immunising antigens were given in strong basic buffers (carbonate) to transiently neutralise the acidic pH of the stomach fluid (85). Alternatively antigen delivery systems such as ISCOMS (Immune stimulating complexes) liposomes, biodegradable microspheres and live recombinant bacteria have therefore been constructed (reviewed in (86)).
The use of recombinant non virulent bacteria provides a potentially powerful method of delivering antigens to the mucosa. The way of antigen presentation by the bacteria is the most controversial aspect in this field. Three possible strategies have been developed. Either the antigen is secreted, produced intracellularly or in a membrane–associated form. Surface displayed heterologous antigens have the advantage that the bacterial outer membrane proteins might mediate an immuno-adjuvant effect (87). Because surface antigens are destroyed during stomach passage it is only possible to elicit an immune reaction if the delivering bacteria have the ability to colonise the intestine. It has been shown, that the immunogenicity is indeed higher when the peptide of interest is genetically fused to a bacterial cell surface component. Several systems for displaying proteins on the surface of both Gram-negative and Gram-positive bacteria have been described until now. It was shown that the recombinant bacterial cells were able to induce humoral immune response after oral vaccination of mice. However in most cases the antibody titer was low and high amounts of bacteria were needed.
Oggioni et al. immunised mice at the same time orally and intranasally with 10^9 recombinant commensal *Streptococcus gordonii* expressing either the M6 protein of *Streptococcus pyogenes* or the E7 protein of human papilloma virus as a fusion with the M6 protein. Less than half of the immunised mice showed a slight immune reaction against the recombinant protein (88-90). The potential of *Streptococcus gordonii* as a vaccine delivery vehicle was highlighted in recent publications, which document prototype vaccines against HIV (91) and measles (92) after subcutaneous immunisations. In another approach used by Nguyen et al. a heterologous receptor (BBG3) was assembled and expressed on the surface of *Staphylococcus xylosus* using signal sequence and cell surface-binding regions of staphylococcal protein A (SpA). Using huge amounts of these recombinant bacteria (24 doses of 10^{10} bacteria) anti-BBG antibodies could be found in three out of four mice after oral immunisation (93). Iwaki et al. found a salivary IgA and a systemic IgG response when mice were immunised orally with recombinant *Streptococcus lactis* (7 times 10^9 formalin-killed bacteria) bearing a surface protein antigen (PAc) from *Streptococcus mutans* serotype c (94).

Oral immunisation using lactic acid bacteria

Because of the widely accepted beneficial effects of lactic acid bacteria (LAB) on human health and their known safety, great interest has been focused on the development of an oral vaccine using this type of bacteria. LAB have been used for centuries in the fermentation and preservation of food and are therefore considered GRAS (generally regarded as safe). This is in contrast to other live vaccine carriers like *Salmonella*, *Escherichia coli* and *Vaccinia* which can not be classified as safe. Furthermore, in contrast to LAB, the latter type of carriers are themselves highly immunogenic which may prevent repetitive use of the carriers in multi-schedule immunisations with the same or other antigens.

Lactobacillus strains are members of the commensal bacterial flora and have a number of properties which make them attractive candidates for oral vaccination purposes. Thus certain lactobacillus species have the capacity to colonise certain regions of the intestinal mucosa which permits, in principle, the induction of a local immune response (73). It was shown, that in mice a mucosal as well as a systemic immune response was obtained against peptides coupled to the surface of lactobacilli after oral administration. This finding and other experiments indicate that certain Lactobacillus species have an immunoadjuvant effect have led to the use of lactobacillus as vehicle for expressing foreign antigens (84, 85).
Several Lactobacillus species are covered with a regular surface layer (S-layer) composed of one single species of protein (S-protein; (95)). Because the S-protein is presented in many copies and is not strongly immunogenic it may be an attractive candidate for fusion with antigenic determinants. Therefore a secretion cassette based on the expression and secretion signals of the S-layer protein (SlpA) from *Lactobacillus brevis* (*Lb.brevis*) was constructed. *E.coli* β -lactamase (Bla) was used as a reporter protein to determine the functionality of the S-layer signals for heterologous expression and secretion in different strains of Lactobacillus. In all strains tested Bla was expressed indicating that the *Lb.brevis* slpA signals can be used for protein production and secretion in LAB (96).
The ability of the recombinant LAB strains expressing foreign antigens to evoke an immune response was tested using different expression systems. Zegers et al (77) used *Lactobacillus casei* to express the protective antigen (PA) of *B.anthracis.* High intracellular expression levels for the PA were obtained. In order to investigate whether the expressed PA could induce an antibody specific response against PA mice were immunised with three sets of three successive daily doses either orally with $5x10^{10}$ or 10^9 live *Lact. casei* or intranasally with $2x10^9$ or $4x10^7$ respectively. In addition one group of mice received intraperitoneally a soluble fraction (100μg Protein) of a cell lysate made from recombinant *Lact. casei.* Mice immunised intraperitoneally elicited a strong immune response against PA, but no specific immune response against PA after oral or nasal immunisations was found.
In another approach Claassen et al (97) coupled Trinitrophenyl (TNP), a hapten, to the surface of *Lactobacillus* via free amino acid residues. TNP itself (by definition) can not induce an immune response, any response against TNP would therefore result from T-cell epitopes provided by proteins from *Lactobacillus*. Mice were immunised twice orally with 50 μg of the heat inactivated form of this engineered *Lactobacilli* on day 0 and 21, and an antibody response was found against TNP. IgM peaked on day 7 whereas IgG gradually increased in time. No antibody response was found against Lactobacilli. In order to look at the Lactobacilli uptake and the routing into the immune system Lactobacilli were labelled with DiI (1,1;-dioctadecyl-3,3,3,3;-tetramethylindocarbocyanine perchlorate) and were orally administered to mice. The results showed that Lactobacilli were readily taken up by M-cells. After 6-12 h Lactobacilli were observed in the dome area of PP but no label was seen after 48 hours in the PP. From that time Lactobacilli could be demonstrated in the macrophage-rich regions of mesenterical lymph nodes (97).

In recent studies the work was focused on using lactobacilli for passive rather than active immunisation. Kruger et al have recently described the use of *Lactobacillus zeae* producing single-chain antibodies (scFv), directed at the streptococcal antigen I/II (SAI/II), for passive immunisation (98). *Streptococcus mutans* is the major pathogen in caries development through the production of lactic acid. Although they have not shown direct evidence of a correctly folded scFv fragment, they were able to show a significant reduction of affected animals in comparison to wild type lactobacilli. A very interesting approach was used by Chang et al (99) to inhibit HIV infectivity using *Lactobacillus jensenii* expressing a functional two-domain CD4. In an *in vitro* system they could show an inhibition of HIV infectivity of HeLa-CD4 cells when coincubated with recombinant lactobacilli. These data suggest that Lactobacilli can be used as a carrier for oral vaccination as well as for passive immunisation. However more work on strain selection and expression of surface proteins in relation to immune response is still needed.

Another approach has been to test the lactic acid bacterium *Lactococcus lactis* (LcL) as a vector for oral vaccination. As it does not belong to the commensal species of lactic acid bacteria it is not adapted for growth and replication in vivo. However desired recombinant antigens can be produced by the bacteria during growth in vitro that can also be targeted to different cellular compartments in which their immunogenicity differs. *L. lactis* is approximately the same size as biodegradable microparticles which are known to be taken up by M cells and to be capable of acting as effective oral vaccine vehicles. Immunogens expressed by recombinant lactococci are presented to the immune system in particulate form and should therefore be less likely induce oral tolerance than soluble antigens. For these reasons, *L.lactis* may be a particularly attractive option for the vaccination of young infants, the elderly and the immunosuppressed, and would broaden the range of available recombinant bacterial vaccination vectors. Chemical inactivation of lactococcal vaccine strains may improve safety further (80). Different expression systems were used by Norton et al (1) to produce Tetanus toxin fragment C (TTFC) either as membrane-anchored protein (fused to proteinase PrtP), as intracellular protein or as a secreted protein which is partly retained within the cell wall. After subcutaneous immunisation of mice with variable amounts of all three lactococcal TTFC expressor strains (3 doses of $5x10^6$-$5x10^8$ bacteria) protection was achieved with all strains when the animals were challenged with lethal doses of Tetanus toxin. Only low amounts of anti-Lactococcus antibodies were induced. Compared in terms of the dose of expressed TTFC required to elicit protection LcL strains expressing TTFC in a membrane –anchored form was significantly (10-20 fold) more immunogenic than the two other strains. In order to see whether Lactococci can be used as antigen delivery system for oral immunisations the Lactococcus strain that produces TTFC intracellularly has been inoculated orally and nasally into mice. No serum antibody response was seen against TTFC after oral immunisation. In contrast to the oral immunisation nasal inoculation of the same strain (10^8 or 10^9 live bacteria) induced a significant antibody response and a protection against a challenge of $20xLD_{50}$ of tetanus toxin.
In a recent study Robinson et al (80) have expressed Tetanus toxin fragment C (TTFC) in LcL, using a constitutive lactococcal expression system that has been assembled in a plasmid vector series designated pTREX. Expression of TTFC by a pTREX1 vector results in the intracellular accumulation of TTFC to approximately 1-3% of soluble cellular protein. Oral immunisation of mice with these recombinant Lactococci (2 sets of 3 successive daily doses of $5x10^9$) resulted in serum IgG titers lower than those following intranasal inoculation. However the protective efficacy after tetanus toxin challenge was of the same magnitude. TTFC specific fecal IgA response could be detected following oral or intranasal immunisation indicating that both systemic and mucosal immunity were induced.

Oral tolerance

Oral tolerance to food antigens and gut flora

Oral tolerance is a form of peripheral tolerance in which mature lymphocytes in the peripheral lymphoid tissues are rendered non functional or hyporesponsive by prior oral administration of antigen. The importance of oral tolerance is highlighted by the fact that most of the population have life long clinical and immunological tolerance both to food antigens and to their gut flora. Food-specific secretory IgA antibodies are generally absent in normal individuals, indicating that mucosal IgA production is regulated in a way similar to that of systemic immunity (4). However a sizeable minority of the population (approximately 5%) suffer from adverse reactions to foods of which 40% are thought to have known immunological basis. Food-allergic diseases are a feature of childhood and may represent a breakdown or failure of oral tolerance induction or maintenance. In childhood, most food-allergic diseases are self-limiting with a highly variable time span. Inflammatory bowel disease may represent a similar breakdown of tolerance to components of the gut flora and foods.

Factors affecting oral tolerance induction

There are three potential outcomes after oral exposure to an antigen, induction of systemic immunological unresponsiveness (tolerance), systemic priming or induction of local secretory IgA responses in the absence of measurable systemic immune responses.
Tolerance can probably be induced to all thymus-dependent soluble antigens, a feature that has hampered the successful development of oral vaccines unless mucosal adjuvants like non-toxic subunits of Cholera-toxin (CT-B) or E.coli-toxin (LT-B) are used (100-102). Requirements for tolerance induction may be different for individual antigens or antigenic mixtures. It seems that high doses of antigen and an intact GI-flora promote tolerance. In contrast low antigen doses, immature host, a defective GI-flora and particulate or replicating antigens often induce active immunity instead of tolerance. Provision of an inflammatory or invasive stimulus also prevents the induction of tolerance, and when antigens such as OVA are coupled to immune-stimulating complexes (ISCOMs) or to bacterial toxins, local and systemic immunity are likely (103).

The most frequent outcome of an oral encounter with soluble antigen is systemic tolerance. Most aspects of the systemic immune response of naive animals can be suppressed by single or multiple fed antigens. Within 24 hours after a single feeding of an antigen regulatory cells have been demonstrated in the PP and mesenteric lymph nodes and in the spleen after 4-7 days. Oral tolerance to systemic challenge is well established within 5-7 days. Thereafter tolerance seems to be long-lasting, with suppression of delayed type hypersensitivity (DTH) being demonstrable for up to 17 months after a single feed (104).
Many different doses and regimens of single and multiple feeds induce oral tolerance successfully. There is some evidence that single administration of high doses of antigen (approximately >0.5mg g^{-1} body weight in mice) induces suppression of virtually all responses by direct inactivation of T cells (103, 105), whereas multiple low doses (<0.1mg g^{-1} body weight in mice) are more likely to generate regulatory cells. Very low doses of antigen (<0.005 mg g^{-1} body weight in mice) given orally have been shown to prime the animal for subsequent systemic and local immune responses (106). In contrast whey proteins from cow's milk administered once at doses equivalent to a tolerising dose of ovalbumin (OVA, 1mg/g body weight) can induce a split tolerance – leading both to suppression of DTH (Th1 suppression) and to priming for IgG and IgE responses (Th2 enhancement) (4).

Animals that have been previously fed with a small amount (100μg) of antigen such as OVA or lysozyme mount a suppressed cellular and humoral immune response after subsequent subcutaneous vaccination with that antigen (5). In contrast to these findings, a single feed of OVA (25mg) mounted a significantly higher serum IgG response against OVA compared with unfed mice after subcutaneous vaccination with OVA emulsified in an oil-based adjuvant (107).
But the status of local mucosal immune responses to orally administered antigens remains controversial. While some groups suggest that the suppression of systemic immunity is accompanied by local secretory IgA production or induction of PP T cells (108, 109) others have reported that intestinal IgA antibody production is reduced (110).

The host immaturity can have great impact on tolerance induction. Intragastric antigen administration to neonatal rodents during the first 7-10 days of life does not suppress systemic immunity and may prime for later systemic immunity and autoimmune responses. It seems that this inability is not merely a result of the immaturity of the digestive system or antigen-handling capacity of the neonatal gut, but is more likely to be due to an as-yet-uncharacterised regulatory imbalance that can be partially restored with adult spleen cells (111).

The amount of antigen absorbed intact varies widely, between 0.001% and 1% of the administered dose, and it seems likely that differences in absorption could influence oral tolerance. But no clear relationship could be demonstrated. Other factors such as site and rate of antigen elimination, as well as the rate and extent of intralumenal antigen digestion have yet to be defined (4).

Mechanisms of oral tolerance induction

There is still much controversial discussion about tolerance induction. Probably multiple mechanisms are involved in this crucial physiological phenomenon. Tolerance is immunologically specific and must therefore be due to the deletion or inactivation of antigen-specific T lymphocytes, as B cells seem not to be important players in oral tolerance (4). Immature or developing lymphocytes are more susceptible to tolerance induction than are mature or functionally competent cells. Whether the recognition of a foreign antigen by lymphocytes results in activation or tolerance depends on the maturational stage of the specific lymphocytes, the nature of the immunological stimulus and (for T cells) the nature of the antigen presenting cells.
Three principal immunological mechanisms have been implicated in oral tolerance: clonal deletion, clonal anergy and antigen-driven suppression. Clonal deletion or cell death is rarely found in peripheral tolerance to nominal antigens and has not been described during oral tolerance induction in normal animals. High doses of antigen (>10mg/dose) given to normal mice are believed to induce clonal anergy. Low doses (below 100μg per dose) seem to induce tolerance by active suppression, mediated by the production of cytokines such as IL-4, IL-10 and TGF-β (5, 112).
It is thought that clonal anergy of T cells is induced by an aberrant presentation of antigen by antigen-presenting cells (APCs) lacking a full range of costimulatory molecules such as CD80/CD86, intercellular adhesion molecule 1 (ICAM-1) and others. There are several candidates for such tolerogenic APC. Orally administered OVA could be found in enterocytes that usually express low amounts of MHC class II. As these cells do not express ICAM-1 or CD80, antigen presentation by these cells might lead to anergy of naive CD4+ cells. (113). Other cell types that may be involved are CD8+ T cells with their CD1 –like epithelial cell ligands or dendritic cells (DC). These findings and the underlying experiments give direct

evidence that anergy rather than deletion is one of the major mechanisms underlying oral tolerance.

Nevertheless there remains substantial evidence of a role for active suppressor mechanisms. Anergic cells for example may act as functional suppressor cells by production of inhibitory mediators or by competing for APCs and growth factors. T cells with suppressive activity have been identified in the intestinal mucosa, mesenteric lymph nodes and spleen. Although this active suppression was believed originally to be a property of CD8+ T cells, more recent studies have suggested that these cells are not necessary for tolerance induction, because in CD8+-knockout mice tolerance can be induced (114). This suggests that CD4+ T cells, maybe a further functional subset termed Th3, that produce Il-4, Il-10 and TGF-β, rather than CD8+ T cells are required for oral tolerance induction. Not only αβ T cells but also T cells expressing the γδ TCR may play an important role in the regulation of specific mucosal immune responses and systemic tolerance by secreting IL-10 (115) and IFN-γ respectively.

Costimulatory molecules are also believed to participate in tolerance induction. Blockade of B7-CTLA4 interactions completely inhibits high-dose induction of systemic tolerance. Similarly it is not possible to induce systemic T cell unresponsiveness to OVA in CD40 ligand gene-disrupted mice (116).

Orally applied bacteria can differentially affect oral tolerance. Maassen et al found, that in experimental autoimmune encephalomyelitis (EAE) in mice different strains of Lactobacillus could either enhance the disease (*Lactobacillus reuteri*) or inhibit the disease to a certain extend (*Lb.casei*, *Lb.murines*). They found, that orally applied strains that tend to enhance EAE mainly induce a Th1 like cytokine profile (TNF-α and IL-2) and strains that inhibit antibody responses correlate with the induction of Th2 cytokines as TGF-β and IL-10 (76).

Future perspectives

The induction of oral tolerance may become an attractive tool for treatment of autoimmune diseases and allergies. One could think of a tolerogenic display system (like commensal bacteria), in such a way that tolerogens can be expressed either intracellularly, secreted or anchored to the surface. Such a system could for example be used for specifically tolerising against antigens in different allergies or autoimmune diseases. Wildner et al (117) have shown that the oral application of highly tolerogenic peptides might be a potent approach for the treatment of autoimmune diseases. In endogenous uveitis, a T-cell mediated autoimmune disease that leads to impairment of visual acuity, an antigenic mimicry between a peptide of uveitis-associated HLA-class I antigen and a peptide of retinal autoantigen occurs. Using the HLA-peptide B27PD they could induce to a certain degree tolerance by orally applying this antigen. Within 6 weeks of oral peptide treatment all patients responded with marked decrease of intraocular inflammation. This work is very promising in the field of oral tolerance induction.
Another practical implication could be the induction of an active suppression mechanism that may induce bystander effects (4). Tolerised T cells secrete suppressive cytokines (e.g. TGF-β) in an antigen-specific fashion but its release into the local microenvironment may then also suppress ongoing immune responses to an unrelated but anatomically co-localised antigen.
This so called bystander suppression is a cornerstone of the therapeutic use of oral tolerance in the treatment of autoimmune diseases via the mucosal route as it may avoid the need to identify and use specific autoantigen.

Lactic Acid Bacteria as delivery system for mucosal vaccination

The aim of our work was to develop vaccines based on the expression of heterologous peptides (mimotopes) and anti-idiotypic single chain Fragment variable (α-IdscFv) on the surface of Lactobacillus johnsonii (Lj) and Lactococcus lactis (MG1363). The proteins were cloned into plasmid pMD112 in such a way that they were linked to the C-terminal 1615 amino acids (as) of the *Lactobacillus bulgaricus* proteinase B (118). Two types of mimotopes were cloned into the NheI and PvuI restriction sites of prtB. One is a mimotope derived from Tetanus toxin recognised by a pool of polyclonal anti-Tetanus antibodies (tetanus-mimotope) and the second is an IgE mimotope in order to obtain mimotope based anti-Tetanus and anti-IgE vaccines respectively. The anti-idiotypic scFv fragments consisted of the variable heavy and light chains of Fab α-Id-B43 (67) linked with either a very flexible $(SerGly_4)_3$ linker (119, 120) in α-IdscFv1, a β-turn promoting linker (121) in α-IdscFv2 or a random linker (122) in α-IdscFv3. According to Figure 10 the IgE mimotopes as well as the anti-idiotypic scFv fragments should therefore lead to an anti-IgE immune response upon immunisation using recombinant LAB expressing either mimotopes or anti-idiotypic scFv fragments on their surface.

The plasmid: pMD112

Plasmid pMD112 (Figure 13) was constructed by cloning the gene for proteinase B (prtB) into vector pNZ124 (123), using PCR technique. pMD112 has a size of 8818 bps. Besides a chloramphenicol resistance the plasmid contains two replication proteins, repA and repC repectively.

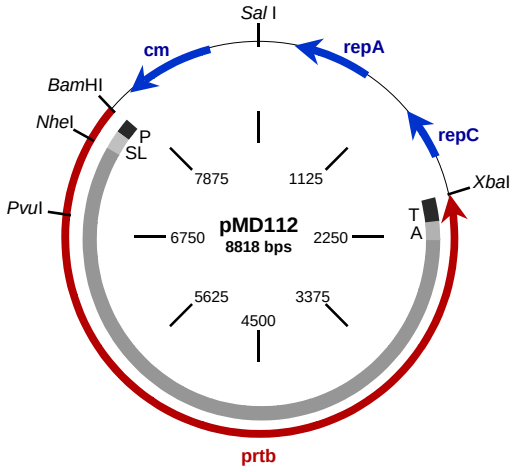

Figure 13: Plasmid pMD112. cm: Chloramphenicol resistance; repA, repC: Replication proteins; P:Promoter; SL:Signal leader; A: Anchor; T: Terminator;

Proteinase PrtB

Originally Proteinase PrtB (Figure 14) has been isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus*. The proteinase may be involved in the specific hydrolysis of caseins. The deduced amino acid sequence shows 1946 residues and a predicted molecular mass of 212 kDa. PrtB is probably synthesised as an inactive preproprotein. The positively charged N-terminal signal peptide (34 residues) is removed during translocation through the cytoplasmic membrane, and the C terminus remains anchored in the cell envelope. The putative cleavage site is most probably located between Ala –159 and Ala –158. After the anchoring process the

proteinase is activated by the removal of the pro region. The cleavage may occur between residues 192 (T) and 193 (D, at position 1). The resulting mature protein would then contain 1754 residues (Figure 14).

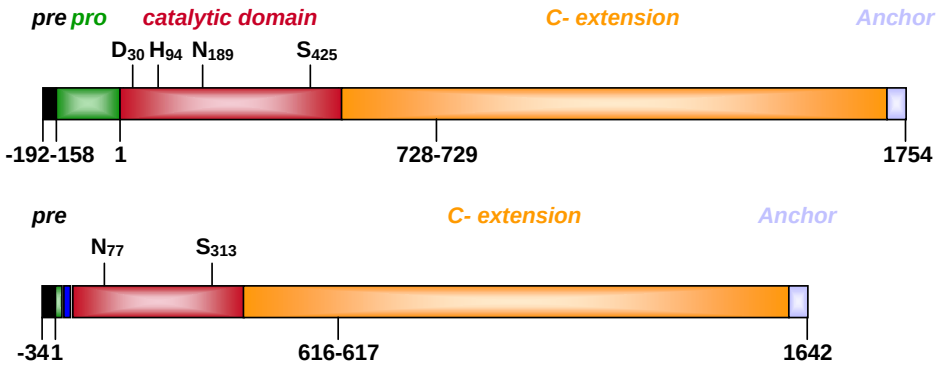

Figure 14: Proteinase PrtB (from Gilbert et al (118))

Predictions to the functionality and structure of PrtB have been obtained by comparing PrtB to PrtP, a lactococcal proteinase (118). The catalytic site of the enzyme involves probably the three amino acids Asp-30, His-94 and Ser-425 as well as asparagine (N-189) that provides electrostatic stabilisation of the oxyanion hole. PrtB is sensitive to cysteine and serine protease inhibitors, so it may be a member of the cysteine-containing subgroup of subtilisin. Probably two regions of PrtB are responsible for substrate binding. The first region lies approximately in the region of aa 130 to 225. Residues 131 (glycine) and 166 (valine) are located on either side of the substrate-binding cleft. The second region lies around amino acids 728 and 729.

Mimotopes and anti-idiotypic ScFv Fragments

Mimotopes are constrained peptides that mimic a part of a conformational epitope of an antigen. Phage display derived peptides isolated by affinity selection from linear or circular libraries are often called mimotopes since they mimic the structure of the original epitope. Upon immunisation, mimotopes have shown to induce an antigen specific immune response against the epitope recognised by the monoclonal antibody (mAb) used for the affinity selection of phage clones (124, 125). Mimotope immunisations could therefore be a way to induce epitope-specific antibody responses in vivo for cases where the complete antigen would be harmful (e.g. toxins) or induce undesired specificities. Table 4 summarises the mimotopes chosen for surface display on PrtB.

Table 4
Mimotopes used for surface display on proteinase PrtB

Name of mimotope	Length (amino acids)	Amino acid sequence
TetMimo101	11	CTDPSGASAPC
Cε3 mimotope	10	VNLPWSFGLE
Cε4 mimotope	10	CINHRGYWVC

Anti-idiotypic single chain Fragment variable mimic part of a conformational epitope of an antigen. They are constructed by linking of the V_H and V_L domains of an anti-idiotypic antibody using a short peptide linker. Upon immunisation, anti-idiotypic scFv fragments have been shown to induce an antigen specific immune response against the epitope recognised by

the monoclonal antibody (mAb) used for the affinity selection (67, 126). Immunisations using anti-idiotypic scFv fragments could therefore be a way to induce epitope-specific antibody responses in vivo in cases where the complete antigen would be harmful (e.g. toxins) and a mimotope would be too small to induce an immune reaction. Table 5 and Figure 15 summarise the chosen anti-idiotypic scFv fragments for surface display on PrtB.

Table 5
Anti-idiotypic ScFv Fragments: Linkers and designations

ScFv Fragment Designation [a]	Linker sequence	Linker description	References for linkers
α-IdscFv1	GGGGSGGGGSGGGGS	Flexible linker	(119, 120)
α-IdscFv2	**PNGA**SNSGSAPDTSSA**PGSQ**	β-turn linker	(121)
α-IdscFv3	YPRSIYIRRRHPSPSLTT	Random linker	(122)

[a] Anti-idiotypic scFv Fragments consist of V_L and V_H of Fab α-Id-B43 as described (67)

Tetanus-mimotope

Tetanus toxoid is a widely used vaccine in humans. Because of the considerable side effects caused by this vaccine Zürcher et al. have tried to develop a better vaccine (127, 128). In order to replace the current tetanus toxoid vaccination by a more specific and better characterised peptide based vaccine circular and linear nonapeptide libraries (CX_9C) displayed on bacteriophage M13 were screened on polyclonal anti-Tetanus toxoid IgG from a pool of about 1000 blood donors. Amongst other mimotopes, the Tetanus-mimotope, termed TetMimo101, was found. It is a circular peptide of 11 amino acids with the primary structure CTDPSGASAPC. The mimotope itself shows sequence homology to amino acids 141-150 of Tetanus toxin fragment A. Although the mimotope is strongly immunogenic it is not protective in a toxin neutralisation assay in mice.

IgE-mimotopes and anti-idiotypic scFv Fragments

In order to develop an anti-IgE vaccine an IgE epitope was defined by screening peptide phage display libraries on a monoclonal anti-IgE antibody. This antibody, termed BSW17, is non-anaphylactogenic, can inhibit IgE synthesis and interfere with the binding of IgE to the high and low affinity receptors for IgE (58). Thus peptides representing the IgE epitope recognised by this mAb may be capable of inducing autoantibodies in vivo with similar characteristics as BSW17. To identify the IgE epitopes specific for BSW17 different phage display libraries that expressed on the surface circular or linear random peptides of nine amino acid length were screened (64). A first mimotope was found which showed a structural homology with a region within the fourth domain of the IgE molecule. As previous results have suggested that BSW17 recognised IgE within the third constant ε3 domain a new set of random peptide libraries were screened. A predominant linear peptide of 10 amino acids was isolated that showed linear sequence homology within the Cε3 domain of IgE. These results suggest that there are probably two IgE binding sites for BSW17. According to their homologies the mimotopes were called Cε4 and Cε3 mimotope respectively.
Both mimotopes coupled to the carrier protein KLH were then used to immunise rhesus monkeys. These monkeys indeed developed strong anti-IgE titers and in vivo passive cutaneous anaphylaxis tests (PCA) have shown that both mimotopes were able to abolish PCA reactivity in the case of allergen challenge (129). Oral immunisation using M13 phages carrying the IgE mimotopes could elicit an immune response against the mimotopes (130).

According to Jerne's network hypothesis, the binding site of an anti-idiotypic antibody also represents the internal image of an epitope present on a foreign, or even a self-antigen (66). Therefore it should be feasible to find anti-idiotypic antibodies with structural homology to isolated random peptides, mimotopes of IgE (Figure 10). To isolate such antibodies, a pool of human Fab phage display libraries were used that had been isolated from peripheral blood mononuclear cells of allergic and non-allergic donors and from purified B cells from children's tonsils. Upon selection on BSW17 two anti-idiotypic antibodies were isolated, that exclusively interacted with BSW17. Interestingly these antibodies mimic the same molecular region as the previously described IgE mimotopes, but they cover a much larger epitope on the IgE molecule (67). Again both anti-idiotypic antibodies were functional in a PCA test in rhesus monkeys. Experiments are continuing to evaluate whether these antibodies show the same long-term protection that was observed with the Cε mimotopes. In a vaccination procedure these anti-idiotypic antibodies may be better tolerated than peptides that have to be coupled to immunogenic carriers.

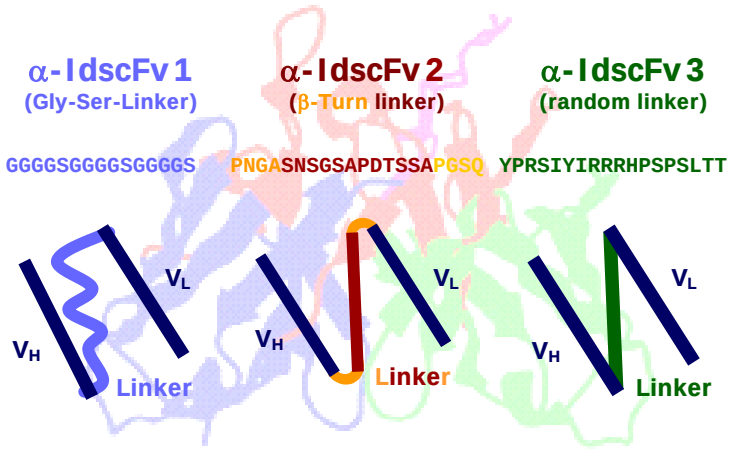

Figure 15: Construction of the anti-idiotypic scFv Fragments

Clinical trials will show whether the results obtained in monkeys are confirmed and whether an anti-IgE immune response will be induced that prevents binding of IgE to FcεRI and consequently the sensitisation of the effector cells involved in allergic disease. However, in order to produce a vaccine based on the use of *Lactobacillus johnsonii* (Lj) as an antigen delivery vehicle, the use of an Fab is not suitable, as gram-positive Bacteria lack a periplasm where heavy and light chain could assemble. Therefore we chose the strategy of an anti-idiotypic single-chain Fragment variable (α-IdscFv).

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Recombinant *Lactobacillus johnsonii* as a mucosal vaccine delivery vehicle

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Abstract

Lactobacilli are considered to be safe organisms making them attractive as vehicles for oral vaccination. We report that *Lactobacillus johnsonii* (Lj) partially survived simulated gastric conditions in vitro, suggesting that it could be used as an oral vaccine delivery vehicle. In order to test this approach, we used the cell wall anchored proteinase PrtB, isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* as a model antigen. Using a new vector system, we demonstrated expression of both proteinase PrtB alone and a mimotope peptide derived from tetanus toxin integrated in the sequence of proteinase PrtB (TTmim–PrtB fusion protein) on the surface of Lj. Oral immunisation of mice with recombinant Lj, expressing the TTmim–PrtB fusion protein induced a systemic IgG response against Lj and recombinantly expressed proteinase PrtB but no antibody response against the tetanus toxin mimotope suggesting that the mimotope was not sufficiently immunogenic to induce an immune response. Interestingly, a proteinase PrtB specific fecal IgA response was also induced, indicating that the proteinase PrtB fusion protein expressed as a cell surface protein on Lj can induce both systemic and local mucosal immune responses.

Keywords: *Lactobacillus johnsonii*; Proteinase B; Tetanus mimotope

1. Introduction

In recent years, new vaccination strategies have been developed in order to avoid disadvantages associated with parenterally administered vaccines [1]. As many viral, bacterial and parasitic pathogens enter the body via mucosal surfaces, oral administration of antigens might simulate the natural route of infection and be a more effective method of immunisation [2]. In addition, oral vaccination applied on a large scale, is relatively inexpensive and frequently induces both local and systemic immune responses, resulting in an effective elimination of pathogens [3].

Lactobacillus strains have a number of properties making them attractive candidates for oral vaccination. Since centuries they have been used for food processing and food preservation and are considered to be safe organisms for human consumption. In addition, some strains of *Lactobacillus* have the ability to colonise certain parts of the intestinal mucosa [3,4]. It has also been observed that particular strains of *Lactobacillus* have an adjuvant effect by enhancing antigen specific immune responses when administered in combination with antigen [5]. Several systems have been described recently using *Lactobacillus* as a carrier for expressing foreign antigens in a form that can be presented to and processed by the immune system of the mammalian host. In a murine model, it has been shown that after oral administration a mucosal as well as a systemic immune response can be achieved against tetanus toxin fragment C produced intracellularly in lactobacilli [6]. These observations prompted us to use one strain of *Lactobacillus*, *Lactobacillus johnsonii* (Lj) for the development of an oral vaccine.

In this study, we analysed the ability of Lj to express heterologous antigens and to act as an antigen delivery vehicle for oral immunisation. As it is probable that antigen associated with the bacterial surface may be more immunogenic [7], a vector termed pMD112 was used to express antigens on the bacterial cell surface. This vector allows the incorporation of the antigen into the cell wall by fusing it to the cell

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wall anchored proteinase PrtB isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* [8]. As model antigens, we used mimotopes, i.e. small, constrained peptides that mimic a portion of a conformational epitope [9,10], and have been shown to monitor an immune response in mice. One of the mimotopes, termed tetanus toxin mimotope (TTmim), is 11 amino acids in length and shows sequence homology to tetanus toxin fragment A (amino acids 141–150). Our data indicate that the TTmim was expressed as a fusion protein with proteinase PrtB on the surface of Lj (TTmim–PrtB) and after oral administration significant anti-PrtB antibodies but no anti-TTmim antibody response were induced.

2. Materials and methods

2.1. Plasmids and oligonucleotides

Plasmid pMD112 was constructed by inserting into pNZ124 [11], the cell wall anchored proteinase *prtB* gene of *L. delbrueckii* subsp. *bulgaricus* (Germond and Delley, unpublished data). TTmim, an 11 mer mimotope recognised by a preparation of affinity purified human anti-TT serum antibodies, was isolated by using random peptide libraries and phage display technology [12]. This mimotope displays homology to amino acids 141–150 of tetanus toxin fragment A. A nonamer mimotope, ε4mim, recognised by the monoclonal mouse anti-human IgE antibody BSW17 [13] has been described elsewhere [14]. Oligonucleotides encoding for TTmim and ε4mim were synthesised by Microsynth GmbH (Balgach, Switzerland) as follows:

TTfwd 5′-CTAGCTGCACAGATCCTTCTGGAGCATCTGCACCTTGCAT-3′;
TTrev 5′-GCAAGGTGCAGATGCTCCAGAAGGATCTGTGCAG-3′;
ε4fwd 5′-CTAGCTGCATTAATCATAGAGGATATTGGGTTTGCAT-3′;
ε4rev 5′-GCAAACCCAATATCCTCTATGATTAATGCAG-3′.

2.2. Bacteria, reagents

Bacterial strains Lj strain NCC2754 and *Lactococcus lactis* strain MG1363 (Lcl) were grown respectively in MRS broth (Difco Laboratories, Detroit, USA), and in M17 broth (Difco) containing 1% glucose. Chloramphenicol (Fluka Chemie AG, Buchs, Switzerland) was used at a concentration of 10 μg/ml. All restriction enzymes were purchased from Axon Lab AG (Baden, Switzerland), Promega (Catalys, Wallisellen, Switzerland) and Boehringer Mannheim (Mannheim, Germany).

2.3. Antibodies and antigens

A polyoxime TTmim construct (polyTTmim) was produced by Keith Rose (University Medical Centre, Geneva, Switzerland) as previously described [15]. Additionally in our construct, a universal T helper epitope (aa 830–843 of tetanus toxin) was C-terminally attached to TTmim. Polyclonal rabbit anti-TTmim serum was produced in our laboratories by immunising rabbits with the polyTTmim construct. A polyclonal rabbit anti-PrtB serum was prepared by immunising a rabbit with *L. delbrueckii* subsp. *bulgaricus* strain ATCC11842 expressing PrtB and was negatively preabsorbed on Lj. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA and HRP-conjugated sheep anti-mouse IgG was purchased from Cappel (ICN, Switzerland). HRP-conjugated goat anti-rabbit antibodies were obtained from Nordic Immunology Laboratories (Tilberg, The Netherlands). Tetanus toxoid (TTd) was obtained from Dr. A.B. Lang (Swiss Serum and Vaccine Institute, Bern, Switzerland) and used at a concentration of 10 μg/ml.

2.4. Generation of Lj and Lcl transformants expressing TTmim– or ε4mim–PrtB fusion protein

Wild type plasmid pMD112 was isolated from transformed LclPrtB using a slightly modified QIAfilter Plasmid Midi Kit (Qiagen AG, Basel, Switzerland). For more efficient lysis, 1 mg/ml lysozyme was added to the lysis buffer and the sample was incubated for 1 h at 37 °C before DNA isolation according to the manufacturer's manual. Plasmid pMD112 was digested with *Pvu*I and *Nhe*I, gel purified and ligated to annealed synthetic oligonucleotides encoding either TTmim or ε4mim, respectively. Ligated DNA was then used to transform Lj and Lcl and plated on either MRS or M-17 plates containing chloramphenicol (10 μg/ml). Clones were then tested for the presence of TTmim or ε4mim DNA by PCR and sequencing analysis (data not shown).

2.5. Gel electrophoresis and Western immunoblots

Transformed bacteria (LjTT, Ljε4) and wild type Lj were grown overnight in 25 ml medium containing 10 μg/ml chloramphenicol. Bacterial cells were harvested by centrifugation at 3000 × *g* for 15 min at 4 °C and washed with 5 ml TBS. Finally, the bacterial pellet was resuspended in 450 μl TBS and 150 μl 4× non-reducing sample buffer (80 mM Tris–HCl pH 6.8, 2.5% SDS, 0.15% glycerol, 0.005% bromophenol blue). A volume of 20 μl aliquots were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (6% acrylamide, 0.5 M Tris–HCl pH 8.8) and run in a 25 mM Tris, 192 mM glycine buffer (pH 8.3) at 100 V for 60 min. The gels were stained with BM Fast stain (Boehringer Mannheim, Germany) or transferred electrophoretically onto Protran BA 83 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After transfer, the membranes were blocked with PBS/5% BSA for 2 h at RT and incubated with either rabbit anti-TTmim serum (1:1000) or anti-PrtB serum (1:2000) overnight at RT followed by incubation for 3 h at RT with a 1:1000 dilution

of HRP-conjugated goat anti-rabbit IgG. Immunoblots were developed with 4-chloro-1-naphtol for 2 min.

2.6. Gastric simulation tests

To test the stability of recombinant LjTT gastric simulation tests were performed using a protocol adapted from Astwood et al. [16]. Approximately 10^{10} LjTT were suspended in 1 ml TBS and 1 ml of 2× SGF solution (126 mM HCl containing 0.6% (w/v)) pepsin purchased from Fluka (Buchs, Switzerland). Incubations were performed at 37 °C in a water bath and samples of 200 μl were quenched by neutralisation with 75 μl 0.16 M Na_2CO_3 at the following time points: 0, 1, 2, 5, 10, 20, 40, 60 and 120 min. Aliquots were plated on agar plates and incubated overnight to determine the titer of surviving bacteria. To test the stability of TTmim–PrtB on the surface of Lj, aliquots of SGF-treated LjTT were coated to wells of an ELISA plate overnight at 37 °C. PrtB was detected using a rabbit anti-PrtB serum (1:1000) followed by an incubation with HRP-conjugated goat-anti-rabbit IgG (Fc) at a dilution of 1:1000.

2.7. Immunisation of mice

A group of three female BALB/c mice (8 weeks old) was fed intragastrically with 2×10^9 live LjTT at days 0, 1, 2 using intubation needles. A booster immunisation was given at days 14, 15, 16. A control group of three female BALB/c mice (8 weeks old) was immunised subcutaneously with 25 μg polyTTmim at days 0 and 14. Sera were collected by retro-orbital bleeding at days 0 and 28, and sera from each of these groups were pooled.

2.8. Evaluation of antibody responses

The antibody response was measured by ELISA. Costar EIA/RIA half-well plates (Costar, Cambridge, MA) were coated overnight at 37 °C with approximately 10^8 wild type Lcl, recombinant LclTT and Lclε4 or with tetanus toxid (TTd) at a concentration of 10 μg/ml. Sera were tested at dilutions of 1:100 and 1:1000 by incubation on the coated bacteria and TTd at 37 °C for 4 h. Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) incubated for 1.5 h at 37 °C. The 3,3′,5,5′-tetramethylbenzidine (Fluka, Buchs, Switzerland) was used as substrate. The absorbance was read after 10 min at 450 nm on a molecular devices ELISA reader (Paul Bucher, Basel, Switzerland). Serum of non-immunised mice as well as the pre-bleed of the immunised mice were used as negative controls (background) and subtracted from serum signals. Absorbance in these samples was never higher than OD 0.1.

2.9. Measurement of fecal IgA responses

The mucosal IgA immune response was measured by ELISA. Costar EIA/RIA half-well plates (Costar) were coated overnight at 37 °C with approximately 10^8 wild type Lj, with LjTT or Ljε4. Fresh fecal pellets (0.1 g) from immunised mice were collected at days 0 and 28 and treated as described elsewhere [17]. Samples were then tested at a dilution of 1:2 by incubation on the coated bacteria at 37 °C for 4 h. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgA incubated for 1.5 h at 37 °C. Tetramethylbenzidine (Fluka) was used as substrate. The absorbance was read after 10 min at 450 nm on a molecular devices ELISA reader (Paul Bucher). Fecal pellets of non-immunised mice as well as the pellets from day 0 of the immunised mice were used as negative controls (background) and subtracted from signals. Absorbance in these samples was never higher than OD 0.1.

3. Results

3.1. Transformation of lactic acid bacteria with pMD112TT and pMD112ε4

In order to develop an oral vaccine based on the use of Lj as an antigen delivery vehicle, the plasmid pMD112 was chosen as expression vector. This plasmid contains the entire structural gene of the cell wall anchored PrtB from *L. delbrueckii* subsp. *bulgaricus*. Annealed complementary oligonucleotides coding for the TTmim, a peptide of 11 amino acids derived from tetanus toxin (unpublished results) and for control purpose the ε4 mimotope (ε4mim), an IgE decapeptide [14] were inserted into the *Nhe*I and *Pvu*I restriction sites which are located in the active site of PrtB. The resulting plasmids pMD112TT and pMD112ε4 thus code for peptides inserted in the sequence of prtB that are expressed as fusion proteins on the bacterial cell surface.

Lj was transformed with the recombinant plasmids pMD112TT and pMD112ε4 (Table 1). For control purposes Lcl was transformed with the same recombinant plasmids. DNA was prepared from chloramphenicol resistant transformants and tested by PCR and sequencing analysis for the presence of the recombinant plasmids using primers specific for the different mimotopes and the wild type PrtB (data not shown).

3.2. Expression of the recombinant TT-PrtB fusion protein in Lj

The expression of the TTmim–PrtB fusion protein in Lj was analysed by SDS-PAGE and Western blots using wild type Lj and recombinant LjTT. Recombinant Ljε4 was used as a control and analysed on the same gels. Fig. 1A shows the presence of protein bands with molecular weights between 90 and 200 kDa in both recombinant Lj clones (lanes 2 and 3) but not in wild type Lj (lane 1). On the Western blot, these bands showed reactivity with rabbit-anti PrtB serum (Fig. 1B), indicating that the PrtB protein was expressed by the Lj bacteria and that these bands

Table 1
Bacterial clones and designations

Clone designation	Bacterial strain	Plasmid used for transformation	Mimotope fused to PrtB	Expressed fusion protein[a]
Lj	*L. johnsonii*	None	None	None
Lcl	*L. lactis*	None	None	None
LclPrtB	*L. lactis*	pMD112	None	PrtB
LjTT	*L. johnsonii*	pMD112TT	TTmim	Ttmim–PrtB
LclTT	*L. lactis*	pMD112TT	TTmim	Ttmim–PrtB
Ljε4	*L. johnsonii*	pMD112ε4	Cε4mim	ε4mim–PrtB
Lclε4	*L. lactis*	pMD112ε4	Cε4mim	ε4mim–PrtB

[a] Expression was monitored by Western blot and ELISA (see Section 3 and Fig. 1).

correspond to full length (molecular weight 200 kDa) and to fragments of PrtB. The bacterial clones were then tested for expression of the TTmim–PrtB fusion protein using rabbit anti-TTmim serum. As shown in Fig. 1C (lane 2) only LjTT bacteria express a protein corresponding to full length PrtB that reacts strongly with the rabbit anti-TTmim serum indicating that the TTmim was expressed as fusion protein to PrtB. No reactivity was observed with either wild type Lj or recombinant Ljε4. Expression of the TTmim–PrtB fusion protein on the LjTT cell surface was confirmed by ELISA. Live recombinant LjTT bacteria were coated onto ELISA plates and shown to react with anti-PrtB and anti-TTmim sera (data not shown). The same reactivity was obtained with LclTT and Lclε4 bacteria when incubated with rabbit anti-PrtB serum and with LclTT bacteria when anti-TTmim serum was used (data not shown).

3.3. Stability of Lj and of surface displayed proteinase PrtB in simulated gastric fluid

Bacteria used for oral immunisations should be able to survive the harsh conditions of the stomach. In order to test the stability of recombinant LjTT clones under such conditions, a simulated gastric fluid (SGF) digestion test [16] was performed. Fig. 2A shows the survival curve obtained for LjTT clones and in comparison for LclTT and for *Escherichia coli* expressed as the number of colony forming units (CFU) after various times of digestion. An appreciable proportion of the lactobacilli survived the simulated gastric conditions up to 20 min and even after 120 min more than 10^4 viable cells were found. In contrast, the number of viable *E. coli* bacterial cells dropped to $<10^3$ after 10 min and after 120 min, no viable cells could be found anymore. In the case of LclTT,

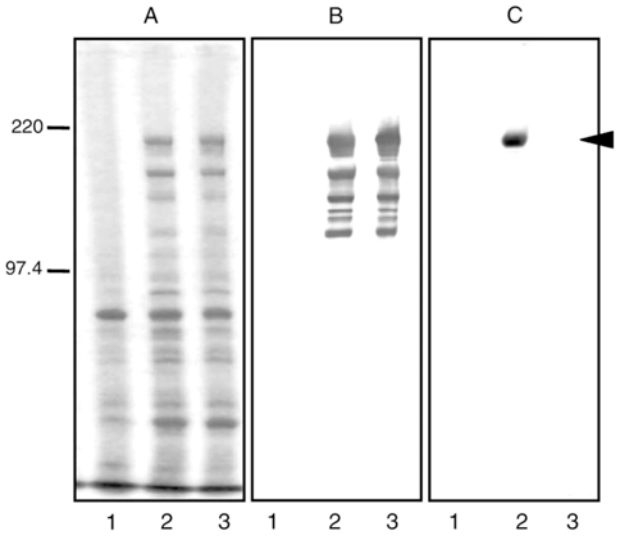

Fig. 1. Recombinant expression of PrtB fusion proteins in Lj. Whole bacterial cell lysates from overnight cultures of Lj (lane 1), LjTT (lane 2) or Ljε4 (lane 3) were separated by SDS-PAGE 6% and stained with BM Fast stain (A) or transferred to nitrocellulose filters and incubated with either rabbit anti-PrtB serum (B) or rabbit anti-TTmim serum (C). Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. The arrow indicates the height of full-length TTmim–PrtB and ε4mim–PrtB.

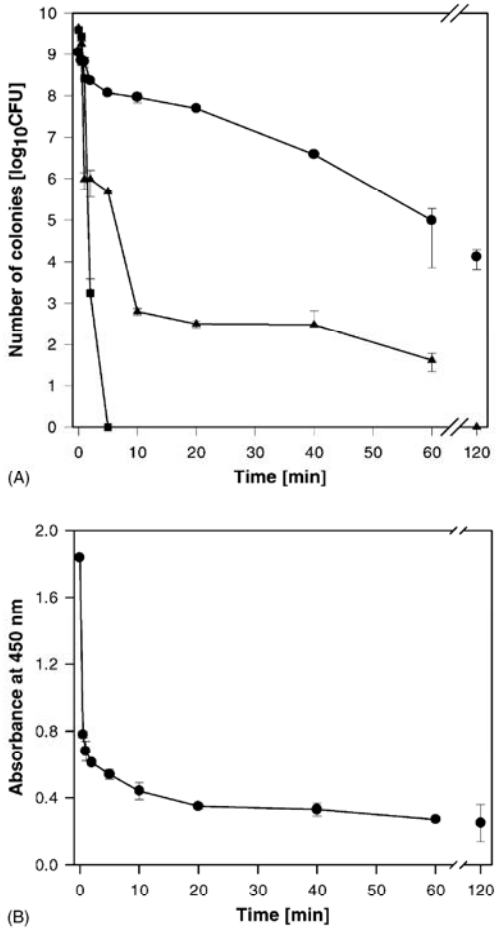

Fig. 2. Survival of LjTT and stability of TTmim–PrtB in SGF. (A) The 10^9 LjTT bacteria (circle) were incubated in tubes containing SGF and digested at 37 °C. At various time points, aliquots were neutralised and streaked out on agar plates. Survival of bacterial cells was measured by counting the CFU. For control purposes 10^9 *E. coli* XL-1 blue (triangle) and 10^9 LclTT (square) were treated the same way. (B) Stability of TTmim–PrtB in SGF was tested by digestion of 10^9 LjTT in tubes containing SGF at 37 °C. At various time points, aliquots were neutralised and coated on ELISA plates. After washing and blocking wells were incubated with rabbit anti-PrtB serum. Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies.

the proportion of viable cells diminished even more drastically and already after 5 min, no viable cells were detected.

To analyse the stability of the cell surface PrtB, an ELISA assay was performed using, as the solid phase, the LjTT bacteria clone after various times of digestion (Fig. 2B). The results, expressed as ELISA OD values, show that almost immediately after exposure (10 s) recombinant Lj bacteria lost reactivity with the anti-PrtB serum. These data indicate that PrtB was digested in the SGF and suggests that in vivo the surface expressed PrtB might not resist gastric digestion.

3.4. Immunisation of mice with LjTT

To investigate whether the expressed TTmim–PrtB fusion protein could induce an antibody response specific for the TTmim and TTd in vivo, groups of mice were fed intragastrically with 10^9 live LjTT at days 0, 1 and 2 and boosted intragastrically at days 14, 15, 16. Sera were tested by ELISA 28 days after the first immunisation using TTd, wild type Lj and recombinant LjTT and Ljε4 clones as coating antigens (Fig. 3). Sera of mice immunised orally with LjTT showed reactivity not only against Lj expressing the TTmim and an unrelated mimotope (ε4mim) but also against wild type Lj bacteria indicating that an antibody response was induced against cell constituents of Lj. In contrast, no reactivity was observed with TTd indicating that no antibody response was induced against TTd after immunisation.

To specifically distinguish between anti-Lj and anti-TTmim–PrtB fusion protein reactivity, sera of mice immunised with LjTT were incubated on ELISA plates coated with wild type Lcl bacteria and recombinant Lcl clones, which also expressed either the TTmim–PrtB or the ε4mim–PrtB fusion protein (LclTT and Lclε4). As shown in Fig. 4, no cross reactive antibodies were observed against wild type Lcl bacteria which do not express a PrtB fusion protein. In contrast, both LclTT and Lclε4 were strongly recognised by sera of mice orally immunised with LjTT. However, according to the Student's *t*-test, no significant difference ($P = 0.08$) between LclTT and Lclε4 was

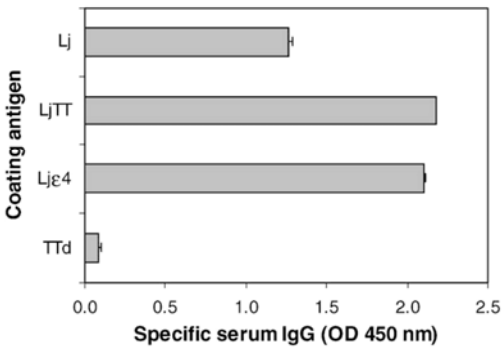

Fig. 3. Binding specificities of serum IgG from mice orally immunised with LjTT. Wells were coated with Lj, LjTT, Ljε4 (approximately 10^8 bacteria per well) or with TTd (10 μg/ml) and incubated with pooled sera collected 28 days after the first immunisation (diluted 1:100). Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) antibodies. Bars represent mean values (±S.D.) for three mice. Lj value is significantly smaller than LjTT ($P < 0.01$) and Ljε4 ($P < 0.01$) values, whereas, there is no difference between LjTT and Ljε4.

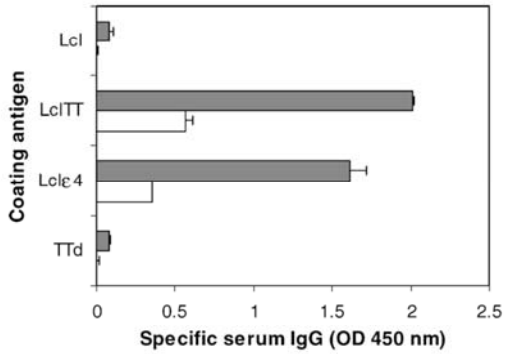

Fig. 4. Detection of serum IgG specific for TTmim–PrtB fusion protein. Pooled sera of mice orally immunised with LjTT react with TTmim–PrtB and ε4mim–PrtB expressed on recombinant Lcl. Wells were coated with wild type Lcl and recombinant LclTT and Lclε4 clones (approximately 10^8 bacteria per well). TTd was coated as a control at a concentration of 10 μg/ml. Wells were incubated with pooled sera collected 28 days after the first immunisation from mice orally immunised with LjTT diluted 1:100 (hatched bars) or 1:1000 (white bars) in PBS/2% FCS/0.05% Tween. Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) antibodies. Bars represent mean values (±S.D.) for three mice. Lcl value is significantly smaller than LclTT ($P < 0.01$) and Lclε4 ($P = 0.01$) values, whereas, there is no difference between LclTT and Lclε4.

observed indicating that the antibody response in the sera was against PrtB and not against the TTmim.

In order to study whether a mucosal immune response had been induced, fresh fecal pellets were collected from mice 28 days after the first oral immunisation with LjTT. Extracts of these fecal pellets were tested for the presence of IgA antibodies against LjTT by ELISA (Fig. 5), using wild

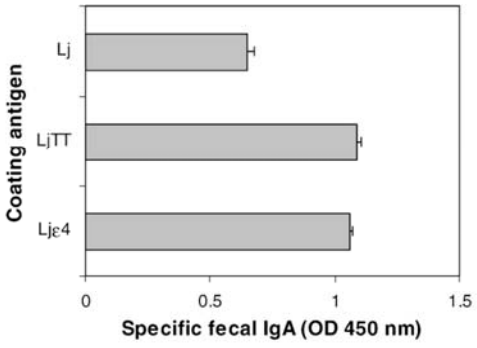

Fig. 5. Binding specificities of fecal IgA from mice orally immunised with LjTT. Wells were coated with different Lj clones and incubated with pooled fecal extracts collected 28 days after the first immunisation diluted 1:2 in PBS. Bound antibodies were detected with HRP-conjugated goat anti-mouse IgA (Fc) antibodies. Bars represent mean values (±S.D.) for three mice. Lj value is significantly smaller than LjTT ($P < 0.01$) and Ljε4 ($P = 0.01$) values, whereas, there is no difference between LjTT and Ljε4.

type Lj and recombinant Lj clones (LjTT and Ljε4) as coating antigens. As shown in Fig. 5, there was an IgA response to both wild type Lj and recombinant Lj expressing either the TTmim–PrtB or ε4mim–PrtB fusion proteins. Based on a Student's *t*-test, significant differences were observed between reactivities to Lj and LjTT ($P = 0.001$) as well as between Lj and Ljε4 ($P = 0.01$). However, there was no significant difference between LjTT and Ljε4 ($P = 0.12$) indicating that the antibody response was directed against PrtB. Thus, LjTT bacteria were able to induce both systemic and mucosal antibody responses against PrtB fusion protein.

4. Discussion

Previous attempts have described the use of certain *Lactobacillus* species as antigen delivery systems for oral immunisation purposes [18]. In this study, we engineered for the first time Lj to express a cell surface antigen, the PrtB of *L. delbrueckii* subsp. *bulgaricus* linked to a TTmim. This recombinant Lj strain was able to induce both mucosal and systemic humoral responses after oral immunisation in mice. Recently, Shaw et al. have also constructed recombinant *Lactobacillus plantarum* and *casei* strains expressing an exogenous antigen, tetanus toxin fragment C (TTFC) for oral immunisation [6]. The antigen was expressed either as an intracellular antigen or a cell surface exposed antigen but this surface exposed TTFC failed to induce a TTFC specific serum IgG. Their results might be explained by the fact that production of heterologous antigen on the cell surface is less efficient compared to intracellular production [19]. In our study, we used a plasmid vector which allows the antigen to be expressed as a fusion protein with the PrtB [8]. Cell surface PrtBs have been isolated from different *Lactobacillus* strains and therefore represent common *Lactobacillus* structures that are well expressed on the surface of the bacteria [20,21]. Thus these PrtBs are ideal candidates to target heterologous proteins to the outer bacterial surface.

Immunisation of mice with recombinant Lj expressing the TTmim–PrtB fusion protein induced PrtB specific IgG serum titers as well as PrtB specific IgA in the feces but no anti-TTmim and anti-TTd antibody responses. The absence of antibody reactivities against TTd may be explained by the fact that the short TTmim (only 11 amino acids long) was not sufficiently immunogenic to compete with proteins and non proteinaceous molecules of larger size present on the surface of the lactobacillus for inducing an immune response. Recently, studies have demonstrated the presence of a non proteinaceous component identified as lipoteichoic acid (LTA) on the surface of Lj with antigenic activity and a molecular mass of 1000 kD [22]. In the present report, the proteinase PrtB, which is a large protein (200 kD) was immunogenic and able to induce both systemic and mucosal responses. Therefore future experiments will be designed to fuse larger antigenic proteins such as anti-idiotypic single chain Fv (scFv), that mimic the epsilon mimotopes, to PrtB.

Recombinant live Gram positive bacteria expressing cell surface protein antigens have already been reported to induce mucosal and systemic immune responses after oral immunisation [23,24]. However, the two strains used in these studies, *Streptococcus gordonii* and *Staphylococcus xylosus*, are not involved in the food chain and are therefore not considered to be safe organisms even though they are commensal organisms and constituents of the human gastro-intestinal flora [25]. Notably in these and other studies using Gram positive commensals, oral immunisations were performed in the presence of bicarbonate solution to compensate for the gastric acid environment [6,18]. We show the induction of an immune response without the addition of bicarbonate solution indicating that Lj bacteria are as previously shown in the SGF test resistant to gastric conditions and survive the gastro intestinal tract. Ingestion of Lj without the use of buffered solutions represents a major advantage for routine application of Lj which could thus be administered alone or as part of a food diet. Additionally serum analysis of the mice indicated a high antibody titer against the constituents of Lj. This confirms other data showing the adjuvant and immunogenic properties of *Lactobacillus* when given parenterally and orally [3,5,26].

Our data show that the PrtB did not survive the SGF conditions but nevertheless an anti-PrtB response was measured in both serum and fecal pellets. This suggests that recombinant Lj bacteria not only acted as passive carriers for the antigen but were able to express PrtB in the gastro intestinal tract. An anti-PrtB response was induced indicating that PrtB was resistant to intestinal enzymes. Recently Zegers et al. have used recombinant *Lactobacillus casei* expressing the protective antigen of *Bacillus anthracis* (PA) for oral immunisation [27]. Their results showed a low *Lactobacillus* specific antibody response and no specific antibody response against PA. However in their system, the PA antigen was expressed intracellularly and in low concentration. Additionally, previous studies have shown that *Lactobacillus* species differ greatly in their properties such as adjuvanticity and persistence in the gut [28]. The fact that we were able to induce antibody responses against both Lj and the whole fusion protein renders Lj functional as an antigen carrier with the appropriate adjuvant properties.

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Dissertation Equivalent B

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Intranasal Immunisation with recombinant *Lactobacillus johnsonii* – a new Strategy to prevent allergic Disease?

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Keywords: *Lactobacillus johnsonii*; anti-idiotypic scFv Fragments; anti-human IgE

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Abstract

We have previously shown a specific anti-IgE response in vivo by parenteral immunisation with short IgE mimotopes or an anti-idiotypic antibody mimicking an IgE epitope. Such an anti-IgE response may be beneficial for the allergic patient. Thus we studied, whether a similar immune reaction can be achieved by mucosal immunisation using live recombinant *Lactobacillus johnsonii* (Lj) as a vaccine delivery vehicle. For surface expression on Lj either an anti-idiotypic scFv or an IgE mimotope was coupled to the cell wall anchored proteinase PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus.* The recombinant Lj were shown to express the heterologous fusion proteins and were specifically recognised by the corresponding anti-human IgE monoclonal antibody. Subcutaneous and intranasal immunisation of mice with recombinant Lj, expressing these fusion proteins induced a systemic IgG response against human IgE. Thus our data suggest that recombinant Lactobacilli expressing IgE epitopes might be used for vaccination to induce a beneficial anti-IgE response as a novel immunotherapy.

1. Introduction

IgE mediated hypersensitivities are emerging in Western Countries. Therefore different approaches aimed at the treatment of allergic patients, independent of the involved antigen, are pursued. A promising approach is the use of non-anaphylactogenic anti-IgE antibodies, that recognise structures close to the binding site of IgE for the high affinity IgE receptor (FcεRI) (1, 2). Such anti-IgE antibodies are presently used for passive immunisation to treat allergic patients (1-8). However, passive immunisation requires continuous treatments with relatively high amounts of monoclonal antibodies. Therefore an active immunisation strategy for neutralising IgE was envisaged by using IgE epitopes to induce a natural autoimmune response, resulting in the formation of autoantibodies that may have the same effect as passively administered non-anaphylactogenic anti-IgE antibodies. To isolate useful immunogenic IgE epitopes a non-anaphylactogenic monoclonal anti-IgE antibody BSW17 was used as screening tool (9, 10) on random phage display libraries (11, 12). Thereby two peptides (Cε3mim and Cε4mim) were isolated mimicking part of the IgE epitope recognised by BSW17 and were therefore called IgE mimotopes. In addition an anti-idiotypic Fab (Fab α-Id-B43) was isolated (13) mimicking the same but larger molecular region as the IgE mimotopes. Both the mimotopes (14) and the anti-idiotypic Fab were used for parenteral immunisation of rhesus monkeys and were shown in a passive cutaneous anaphylaxis (PCA) test to completely abolish PCA reactivity after allergen challenge (manuscript in preparation).

Here we analysed the ability of *Lactobacillus johnsonii* (Lj) to express Cε4mim and α-IdscFv2, an anti-idiotypic single chain Fragment variable (scFv) derived from Fab α-Id-B43 to induce an anti-IgE response upon active, mucosal immunisation. Several systems have been described using *Lactobacillus* as a carrier for expressing foreign antigens and for mucosal immunisation. Zegers et al have reported the use of *Lactobacillus casei* to express the protective antigen of *Bacillus anthracis* as an oral prototype vaccine against anthrax (15). In addition the use of tetanus toxin fragment C (TTFC) as a model antigen for expression on *Lactobacillus plantarum* (16, 17) or on *Lactobacillus casei* (18) and the induction of an anti-TTFC specific immune response after mucosal immunisation has been described. We reported the use of Lj to express mimotopes fused to the cell wall anchored proteinase PrtB (19) and the induction of a local and a systemic immune response against these heterologous proteins after oral immunisation (20).

In the present study we show that both an anti-idiotypic scFv and an IgE Mimotope were expressed as a fusion protein with PrtB on the surface of Lj and induced anti-IgE antibodies

after subcutaneous and intranasal administration. In contrast to other reports we show here that gram-positive bacteria expressing anti-idiotypic single chain Fragment variable (scFv) may be used to act as immunomodulators.

2. Materials and Methods

2.1. Plasmids and Oligonucleotides

Plasmid pComb3His was reconstructed based on pComb3H (12) by inserting 6His tags at the SpeI/NheI sites. Plasmid pComb3-α-Id-B43 contains the sequence encoding for Fab α-Id-B43. Plasmid pMD112 was constructed by inserting into pNZ124 (21) the cell wall anchored proteinase (prtB) gene (19) of *Lactobacillus delbrueckii* subsp *bulgaricus* and was isolated from transformed *Lactococcus lactis* as described recently (20). The linker sequences used for the construction of the anti-idiotypic scFv fragments were designed as described elsewhere (Table 1 and (22-25)). All oligonucleotides were synthesised by Microsynth GmbH (Balgach, Switzerland).

2.2 Bacteria, reagents

Bacterial strain XL-1 Blue was purchased from Stratagene (San Diego, USA). *Lactobacillus johnsonii* strain NCC2754 (Lj) were grown in MRS broth (Difco Laboratories, Detroit, USA) containing 1% Glucose. Chloramphenicol (Fluka Chemie AG, Buchs, Switzerland) was used at a concentration of 10 μg/ml. All restriction enzymes were purchased from Roche Diagnostics GmbH (Rotkreuz, Switzerland).

2.3. Antibodies and antigens

Affinity-purified rabbit anti-PrtB antibodies were purchased from Eurogentech (Seraing, Belgium). Rabbit anti-human ε4mim (10) and anti-human IgE antibody BSW17 (9, 26) were prepared in house as previously described. Rabbit anti-α-Id-B43 was prepared in our laboratory by immunising rabbits using Fab α-Id-B43 (13). Horseradish peroxidase (HRP) conjugated sheep anti-mouse IgG was purchased from Cappel (ICN, Switzerland). HRP-conjugated goat anti-rabbit antibodies were obtained from Nordic Immunology Laboratories (Tilberg, Netherlands). Human myeloma IgE Savasal IgE was a kind gift from Dr. V Savasal (Pilsen, Czech Republic) and was purified on a MonoQ ion exchange column using an FPLC system (Pharmacia Biotech AG, Dübendorf, Switzerland) and used at a concentration of 10 μg/ml.

2.4. Production of anti-idiotypic scFv Fragments in E.coli XL-1 Blue

V_H and V_L sequences of Fab α-Id-B43 (13) and the linker sequences used for the construction of the anti-idiotypic scFv fragments (22-25) were published elsewhere and are shown in Table 1. The V_H and V_L sequences of the anti-idiotypic scFv fragments were amplified separately from Plasmid pComb3-α-Id-B43 using primer pairs specific for the N- and C-terminal of either the V_H or the V_L regions. To assemble V_H and V_L fragments overlapping oligonucleotides encoding different linkers (Table 1) were inserted between heavy and light chain in a PCR reaction. To be used for subsequent cloning the assembled products were PCR amplified with flanking primers containing SacI and SpeI restriction sites.

The resulting DNA fragments encoding either α-IdscFv1, α-IdscFv2 or α-IdscFv3 were then digested with SacI and SpeI restriction enzymes and inserted into plasmid pComb3His digested with SacI and SpeI. Ligated plasmid DNA was used to transform E.coli XL-1 Blue. Positive clones were tested for the presence of the corresponding α-IdscFv DNA by PCR and sequencing analysis (data not shown) and were subjected to large-scale production. Transformed XL-1 Blue were cultivated in 1000ml of super broth (SB) at pH 7.0 in the presence of ampicillin (50µg/ml) at 37°C until an OD at 600nm of 1.0 was reached. Subsequently, the protein synthesis was induced by 1mM IPTG (isopropyl-β-D-thiogalactopyranoside; Promega, Madison, Wisconsin). The bacteria were further cultured by shaking (220rpm) for 4h at 37°C. The culture supernatant was collected and purified on a BD TALON^{TM} Metal Affinity Resin (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

2.5. Screening of anti-idiotypic scFv Fragments by anti-human IgE antibodies

The produced anti-idiotypic scFv fragments were tested by ELISA. Costar EIA/RIA half-well plates (Costar, Cambridge, MA) were coated for 4 h at 37°C with α-IdscFv1, α-IdscFv2 or α-IdscFv3 at a concentration of 10 µg/ml. As a control IgE Savasal was coated at the same concentration. Wells were blocked for 2 h at 37°C with PBS/5%BSA and incubated with either BSW17 (50µg/ml), rabbit anti-human ε4mim (10µg/ml) or rabbit anti-α-Id-B43 (1:1000) for 4 h at 37°C. Bound antibodies were detected by using HRP-conjugated sheep anti-mouse IgG (Fc) or HRP conjugated goat anti-rabbit IgG (Fc) antibodies incubated for 1.5 h at 37°C. Tetramethylbenzidine (3,3',5,5'-Tetramethylbenzidine; Fluka, Buchs, Switzerland)

was used as substrate. The absorbance was read after 5 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher, Basel, Switzerland).

2.6 Generation of Lj transformants expressing PrtB fusion proteins

Recombinant Lj expressing either a Tetanus mimotope (LjTT) or a Cε4mim (Ljε4), recognised by the monoclonal mouse anti-human IgE antibody BSW17 (9) have been described elsewhere (20). To produce recombinant Lj expressing a α-IdscFv2-PrtB fusion protein (Ljα-IdscFv2), DNA encoding α-IdscFv2 was subjected to a PCR reaction using primers containing PvuI and NheI restriction sites. Plasmid pMD112 was digested with PvuI and NheI, gel purified and ligated to the PCR product encoding α-IdscFv2. Ligated DNA was used to transform electrocompetent Lj and transformants were plated on MRS plates containing chloramphenicol (10 μg/ml). Clones were tested for the presence of α-IdscFv2 DNA by PCR and sequencing analysis (data not shown).

2.7. Gel electrophoresis and Western immunoblots

Transformed Ljα-IdscFv2 and LjTT bacteria were grown overnight in 25 ml medium containing 10 μg/ml chloramphenicol. Bacterial cells were harvested by centrifugation at 3000 × g for 15 min at 4°C and washed with 5 ml TBS. After recentrifugation, the bacterial pellet was resuspended in 450 μl TBS and 150 μl ×4 non-reducing sample buffer (80mM Tris/HCl pH 6.8, 2.5% SDS, 0.15% Glycerol, 0.005% Bromophenol blue). 20 μl aliquots were analysed by SDS- polyacrylamide gel electrophoresis (SDS-PAGE) (6% acrylamide, 0.5 M Tris-HCl pH 8.8) and run in a 25 mM Tris, 192 mM glycine buffer (pH 8.3) at 100V for 60 min. The gels were stained with Bio-Safe Coomassie Stain (Bio-Rad Laboratories, Reinach, Switzerland) or transferred electrophoretically onto Protran BA 83 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After transfer the membranes were blocked with PBS/5%BSA for 2 h at RT and incubated with affinity purified rabbit anti-PrtB antibodies (1:2000) overnight at RT followed by incubation for 3 h at RT with a 1:1000 dilution of HRP-conjugated goat anti-rabbit IgG. Immunoblots were developed with 4-chloro-1-naphtol for 2 minutes.

2.8. Catching ELISA with Ljα-IdscFv2

Transformed bacteria (Ljα-IdscFv2, Ljε4 and LjTT) and wild type Lj were grown overnight in 50 ml medium containing 10 μg/ml chloramphenicol. Bacterial cells were

harvested by centrifugation at 3000×g for 15 min at 4°C, washed with 5 ml TBS and recentrifuged. Finally, the bacterial pellet was resuspended in 1 ml TBS/10%(v/v) PBS/2%FCS/0.05%Tween20. 50 µl of this bacterial suspension (approximately 5×10^7 cells) were overlain on Costar EIA/RIA half-well plates (Costar, Cambridge, MA) coated with 50µg/ml anti-human IgE BSW17 overnight at 4ºC. Unbound bacteria were washed off the wells using PBS/0.1%Tween20. Wells were then incubated with rabbit anti-PrtB antibodies diluted 1:2000 in PBS/2%FCS/0.05%Tween20 for 4 h at 37ºC. Bound antibodies were detected using HRP conjugated goat anti-rabbit IgG (Fc) antibodies. Tetramethylbenzidine (Fluka) was used as substrate. The absorbance was read after 5 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher).

2.9. Immunisation of mice

Groups of five female BALB/c mice (8 weeks old) were immunised subcutaneously with 10^8 live LjTT, Ljε4 or Ljα-IdscFv2 at days 0, 21 and 43. The bacterial suspension used for the immunisation was mixed 1:2 with $Al(OH)_3$. Three other groups of five mice were immunised intranasally with three weekly doses of 10^9 live LjTT, Ljε4 or Ljα-IdscFv2 at days 0,1 and 2. Booster immunisations were given at days 21, 22, 23 and 43, 44, 45. Sera were collected by retro-orbital bleeding at days 52 (subcutaneous immunisations) and 56 (intranasal immunisations) respectively.

2.10. Evaluation of antibody responses

The antibody response was measured by ELISA. Costar EIA/RIA half-well plates (Costar, Cambridge, MA) were coated overnight at 37°C with either 10µg/ml human IgE Savasal or 10µg/ml soluble α-IdscFv2. Sera were tested at dilutions of 1:50, 1:100 and 1:200 by incubation on the coated wells at 37°C for 4 h. Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) incubated for 1.5 h at 37°C. Tetramethylbenzidine (Fluka) was used as substrate. The absorbance was read after 6 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher). Serum of non-immunised mice were used as negative controls (background) and subtracted from serum signals. Absorbance in these samples was never higher than OD 0.1.

3. Results

3.1. Production of soluble anti-idiotypic scFv Fragments in E.coli XL-1 Blue

Recently we have isolated an anti-idiotypic Fab (Fab α-Id-B43), mimicking part of the 3rd and 4th constant domains of human IgE (13). In order to develop a mucosal anti-IgE vaccine we intended to express Fab α-Id-B43 on the surface of Lj. Because gram-positive bacteria lack a periplasm where heavy and light chain of an Fab might assemble, the binding site of Fab α-Id-B43 was constructed as a single chain fragment variable (α-IdscFv).

As the linker used for the construction of a scFv influences its correct folding (24, 25), three anti-idiotypic scFv fragments were tested using different linkers. Therefore annealed overlapping oligonucleotides encoding the different linkers for α-IdscFv1 ($(Gly_4\text{-}Ser)_3$-linker), α-IdscFv2 (β-turn-promoting linker, (24)) or α-IdscFv3 (random linker, (25)) were used for assembly of V_H and V_L of α-Id-B43 in a PCR reaction (Table 1). In a subsequent PCR reaction restriction sites were added and the final PCR products encoding the three α-IdscFv1, 2 and 3 were inserted into the SacI and SpeI restriction sites of pComb3His, which contains 6His tags. The recombinant plasmids were transformed into electrocompetent E.coli XL-1 Blue. Soluble anti-idiotypic scFv fragments were produced and isolated on a nickel affinity column.

3.2. Screening for correctly folded anti-idiotypic scFv Fragments

To analyse the isolated anti-idiotypic scFv fragments an ELISA was performed using α-IdscFv1, α-IdscFv2 and α-IdscFv3 as coating antigens. Human IgE was used as a control and was analysed on the same ELISA plate. Fig. 1 shows the recognition of the anti-idiotypic scFv fragments by different antibodies reacting with human IgE. α-IdscFv2, α-IdscFv3 and the control IgE were well recognised by the monoclonal anti-human IgE BSW17 and slightly recognised by the rabbit anti-human ε4mim serum, whereas the recognition of α-IdscFv1 was only minimal. In contrast the rabbit anti-α-Id-B43 serum (BSW17-like) only recognised α-IdscFv2 and the control IgE, but not α-IdscFv1 and α-IdscFv3. As the control IgE showed the same pattern of recognition as α-IdscFv2, only α-IdscFv2 was used in the further experiments.

3.3. Transformation of Lactic acid bacteria

For development of an oral vaccine using *Lactobacillus johnsonii* (Lj), the plasmid pMD112 was chosen as expression vector. This plasmid contains the entire structural gene of the cell wall anchored proteinase (PrtB) from *Lactobacillus delbrueckii* subsp. *bulgaricus.* DNA encoding α-IdscFv2 was amplified in a PCR using primers containing PvuI and NheI restriction sites. The final PCR product coding for α-IdscFv2 was inserted at the NheI and PvuI restriction sites which are located in the active site of PrtB. In addition an ε4 mimotope (Cε4mim), an IgE decapeptide (10) and for control purpose a TT mimotope (TTmim), a peptide of eleven amino acids derived from Tetanus toxin were used, which have been cloned in the same way as α-IdscFv2 as described recently (20). The resulting plasmids pMD112α-IdscFv2, pMD112ε4 and pMD112TT thus code for proteins inserted into the sequence of prtB that are expressed as fusion proteins on the bacterial cell surface.

Lj was transformed with the recombinant plasmids pMD112α-IdscFv2, pMD112ε4 and pMD112TT (Table 2). DNA was prepared from chloramphenicol resistant transformants and tested by PCR and sequencing analysis for the presence of the recombinant plasmids using primers specific for the different peptides and the wild type proteinase PrtB (data not shown).

3.4. Expression of the recombinant PrtB fusion proteins in Lj

The expression of the α-IdscFv2-PrtB fusion protein in Lj was analysed by SDS PAGE Western blot and ELISA. Both Ljε4 and LjTT were already shown to express recombinant PrtB fusion proteins (20) and were used as controls in addition to wild type Lj. Fig. 2A shows the presence of protein bands with molecular weights between 90 to 210 kDa both in recombinant LjTT and Ljα-IdscFv2 clones. On the Western blot these bands showed reactivity with rabbit-anti PrtB serum (Fig. 2B), indicating that the PrtB protein was expressed by the Lj bacteria and that these bands correspond to full length (MW 190 kDa for LjTT and 212 kDa for Ljα-IdscFv2) and to fragments of PrtB. As α-IdscFv2 is a conformational epitope, the bacterial clones were tested for correctly folded α-IdscFv2-PrtB fusion protein in a catching ELISA. BSW17 was coated onto ELISA plates and was overlaid with either Lj, LjTT, Ljε4 or Ljα-IdscFv2. Unbound bacteria were washed off the wells, and bound bacteria were detected using rabbit anti-PrtB antibodies. As shown in Fig. 2C (3rd and 4th bar) only Ljε4 and Ljα-IdscFv2 bound to BSW17 coated on the solid phase, indicating that α-IdscFv2 was expressed as fusion protein to PrtB. Only a marginal reactivity was observed with either wild type Lj or recombinant LjTT.

3.5. Subcutaneous Immunisation of mice with Ljα-IdscFv2

Sera of mice immunised subcutaneously with wild type and recombinant *Lb. johnsonii* bacteria were tested by ELISA 52 days after the first immunisation using soluble α-IdscFv2 (Fig. 3A) or human myeloma IgE ("Savazal") on the solid phase (Fig. 3B). Fig. 3A shows that different dilutions of sera of mice immunised with Ljα-IdscFv2 recognise soluble α-IdscFv2. Weaker reactivities to soluble α-IdscFv2 were also observed with sera of mice immunised either with Ljε4 or with the control strain LjTT. According to a student's *t*-test the reactivities observed with sera of mice immunised with either Ljα-IdscFv2 or Ljε4 were significantly different from the ones observed with sera of mice immunised with LjTT. This results indicate that a specific response was induced against α-IdscFv2 after immunisation with either Ljα-IdscFv2 or Ljε4. As shown in Fig. 3B sera of mice immunised with Ljα-IdscFv2 and Ljε4 also react with human IgE. However only the reactivity observed with the sera of mice immunised with Ljα-IdscFv2 was statistically different from the one obtained with sera of mice immunised with LjTT. Thus a specific reaction against IgE was induced only upon immunisation of mice with Ljα-IdscFv2.

3.6. Intranasal Immunisation of mice with Ljα-IdscFv2

Sera of mice immunised intranasally with wild type and recombinant *Lb. johnsonii* bacteria were tested by ELISA 52 days after the first immunisation using soluble α-IdscFv2 (Fig. 4A) or human IgE Savazal on the solid phase (Fig. 4B). As shown in Fig. 4A soluble α-IdscFv2 reacts with sera of mice immunised intranasally with Ljα-IdscFv2 but also to a lesser extent to sera of mice immunised with either Ljε4 or the control strain LjTT. Based on the Student's *t*-test, only sera of mice immunised with either Ljα-IdscFv2 or Ljε4 were reacting specifically to soluble α-IdscFv2. The results obtained with sera tested at different dilutions indicate that Ljα-IdscFv2 induces a stronger specific antibody response to α-IdscFv2 than Ljε4. In contrast Fig. 4B shows that human IgE was stronger recognised by sera of mice immunised with Ljε4 than with Ljα-IdscFv2 indicating that intranasally Ljε4 induce a stronger anti-IgE response than Ljα-IdscFv2. Sera of mice immunised with the control strain LjTT also reacts weakly with human IgE but according to a Student's *t*-test reactivities to IgE were specific for sera immunised with either Ljα-IdscFv2 or Ljε4.

4. Discussion

Previously we described the use of *Lactobacillus johnsonii* (Lj) as a mucosal vaccine delivery vehicle. As a model antigen we used the cell surface proteinase PrtB isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus*. This model antigen proofed to be immunogenic after intragastric delivery of recombinant Lj (20). Here we engineered Lj to express PrtB linked to either an anti-idiotypic single chain fragment variable (α-IdscFv2), or a Cε4 mimotope (Cε4mim). Both the Cε4mim and the binding site of α-IdscFv2 were recognised by the protective monoclonal anti-IgE antibody, BSW17 and were able to induce a systemic anti-human IgE antibody response after subcutaneous and intranasal immunisation.

Furthermore we used our anti-idiotypic Fab (Fab α-Id-B43) mimicking the epitope on human IgE as recognised by BSW17 (13). Parenteral immunisation of rabbits using Fab α-Id-B43 resulted in the induction of high-affinity antibodies with the same characteristics as BSW17. In order to target an antibody molecule onto the surface of Lj, the binding site of Fab α-Id-B43 had to be constructed as a single chain Fragment variable (scFv). However, many examples in the literature described scFv's which were unstable or had suboptimal binding properties (27-29). In addition it is also known, that the nature of the linker may have an influence on the proper folding of a scFv (30, 31). Therefore three different linkers were tested for holding the variable heavy and light chain regions of Fab α-Id-B43 together. The linker of α-IdscFv2, bearing a β-Turn motif on both ends (24), proofed to be the most appropriate for cloning the anti-idiotypic scFv into PrtB as it was the only scFv recognised by both BSW17 and the rabbit anti- Fab α-Id-B43 (BSW17-like) antibodies.

We engineered for the first time Lj to express an anti-idiotypic scFv (α-IdscFv2) linked to the cell surface protease PrtB. Recently other reports have also described the use of Gram-positive bacteria as carriers for the expression of scFv proteins. Gunneriusson et al have reported the use of Staphylococci to surface display scFv antibodies in order to develop a whole-cell diagnostic device and for using as an alternative for filamentous phages in scFv libraries (32). More recently *Lactobacillus zeae* producing scFv antibodies, recognising the streptococcal antigen I/II were used to deliver passive immunity to *Streptococcus mutans* in a rat model of caries development (33). However, in contrast to these studies we were interested to use the recombinant Lj for active immunisation. Thus our study is the first report of an anti-idiotypic antibody fragment on recombinant gram-positive bacteria that can be used for active immunisation.

Both recombinant bacterial clones (Ljα-IdscFv2 and Ljε4) induced after subcutaneous or intranasal immunisation a specific anti-IgE response. However the magnitude of the anti-IgE response was strongly influenced by the route of immunisation and was depending on the recombinant bacterial clone used. Upon subcutaneous immunisation the α-IdscFv2 expressing clone (Ljα-IdscFv2) induced a higher anti-IgE immune response than the Cε4mim expressing clone (Ljε4) indicating that the α-IdscFv2 is more immunogenic than the small Cε4mim. However, after intranasal immunisation the opposite was the case. This is consistent with the data obtained by oral delivery of phage particles expressing the same antigens (34). In this study Cε4mim expressing M13 bacteriophages also induced a higher anti-IgE titer than Fab α-Id-B43 expressing phages. These results might be explained by the more complex conformational structure of the anti-idiotypic antibody fragments in comparison to the Cε4mim. To be folded correctly anti-idiotypic antibodies are therefore more dependent on the route of immunisation where different conditions prevail. The nasal route of immunisation is thought to be especially restrictive for antibody structures, as the nasal cavity is known to harbour proteases specific for antibody structures (35). Such proteases, produced by resident aerobic bacteria (36), might lead to the digestion of α-IdscFv2.

We show here after intranasal immunisation with recombinant Lj, expressing IgE epitopes, the induction of an anti-IgE response. The question remains however, whether these antibodies are protective and may reduce allergic symptoms in atopic patients. Monkey immunisations using soluble Cε4mim or Fab α-Id-B43 have shown that both IgE epitopes behaved as surrogate antigens and were able to induce an anti-IgE response capable to inhibit an in vivo passive cutaneous anaphylaxis test (37). Thus, it can be expected that also recombinant Lj bearing the same antigens on their surface can induce a qualitatively similar anti-IgE immune response which would prevent binding of IgE to the FcεRI, and consequently inhibit the sensitisation of the effector cells implicated in allergic disease.

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Tables

Table 1

Linkers for the construction of anti-idiotypic scFv fragments

ScFv designation[a]	Linker sequence	Linker description	Reference for linkers
α-IdscFv1	GGGGSGGGGSGGGGS	Flexible linker	(22, 23)
α-IdscFv2	**PNGA**SNSGSAPDTSSA**PGSQ**	β-turn linker	(24)
α-IdscFv3	YPRSIYIRRRHPSPSLTT	Random linker	(25)

[a] Sequences of V_L and V_H of Fab α-Id-B43 are already published (13)

Table 2

Bacterial clones and designations

Clone designation	Bacterial strain	Plasmid used for transformation	Peptide fused to PrtB	Expressed fusion protein[a]
Lj	*Lactobacillus johnsonii*	none	none	none
LjTT	*Lactobacillus johnsonii*	pMD112TT	TTmim	TTmim-PrtB
Ljε4	*Lactobacillus johnsonii*	pMD112ε4	Cε4mim	ε4mim-PrtB
Ljα-IdscFv2	*Lactobacillus johnsonii*	pMD112α-IdscFv2	α-IdscFv2	α-IdscFv2-PrtB

[a] Expression was monitored by Western blot and ELISA (see Results, Fig. 2 and (20))

Figure Legends

Fig. 1

Detection of the anti-idiotypic scFv fragments by anti-human IgE Antibodies. Wells were coated with α-IdscFv1, α-IdscFv2, α-IdscFv3 or with human IgE Savasal at a concentration of 10 μg/ml. Wells were subsequently incubated with either 50μg/ml of monoclonal mouse anti-human IgE (BSW17, white bars), with a rabbit anti-human ε4mim serum (diluted 1:1000, grey bars) or a rabbit anti-α-Id-B43 serum (BSW17-like, diluted 1:1000, black bars). Bound antibodies were detected by using either HRP-conjugated sheep anti-mouse IgG (Fc) or HRP-conjugated goat anti-rabbit antibodies. Bars represent mean values (± SD) from three individual experiments.

Fig. 2

Recombinant expression of α-IdscFv2-PrtB fusion protein in Lj. (A,B) Whole bacterial cell lysates from overnight cultures of LjTT (lane 1) or Ljα-IdscFv2 (lane 2) were separated by SDS-PAGE 6% and stained with Bio-Safe Coomassie Stain (A) or transferred to nitrocellulose filters and incubated with affinity purified rabbit anti-PrtB antibodies (B). Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. The arrow indicates the height of full-length α-IdscFv2-PrtB. (C) Wells were coated with BSW17 (anti-human IgE) at a concentration of 50μg/ml and overlaid with 5 x 10^7 wild type Lj (Lj wt), or recombinant Lj bearing either a Tetanus derived mimotope (LjTT), an IgE (Cε4) derived mimotope (Ljε4) or α-IdscFv2 fused to PrtB (Ljα-IdscFv2). Unbound bacteria were washed off and wells were incubated with affinity purified rabbit anti-PrtB antibodies. Bound antibodies were detected with HRP conjugated goat anti-rabbit IgG (Fc) antibodies. Bars represent mean values (± SD) from three individual experiments.

Fig. 3

Binding specificities of serum IgG from mice subcutaneously immunised with Ljα-IdscFv2, Ljε4, or LjTT. Wells were coated with either 10μg/ml soluble α-IdscFv2 produced in Escherichia coli XL-1 Blue (A) or with human IgE Savasal at a concentration of 10μg/ml (B). Wells were incubated with different dilutions of sera collected from mice immunised subcutaneously with either Ljα-IdscFv2 (triangles), Ljε4 (squares) or LjTT (circles). Bound

antibodies were detected using HRP-conjugated sheep-anti-mouse IgG (Fc) antibodies. Values represent mean values (±S.D.) for five mice.

Fig. 4

Binding specificities of serum IgG from mice intranasally immunised with Ljα-IdscFv2, Ljε4, or LjTT. Wells were coated with either 10μg/ml soluble α-IdscFv2 produced in Escherichia coli XL-1 Blue (A) or with human IgE Savasal at a concentration of 10μg/ml (B). Wells were incubated with different dilutions of sera collected from mice immunised intranasally with either Ljα-IdscFv2 (triangles), Ljε4 (squares) or LjTT (circles). Bound antibodies were detected using HRP-conjugated sheep-anti-mouse IgG (Fc) antibodies. Values represent mean values (±S.D.) for five mice.

Figures

Fig. 1

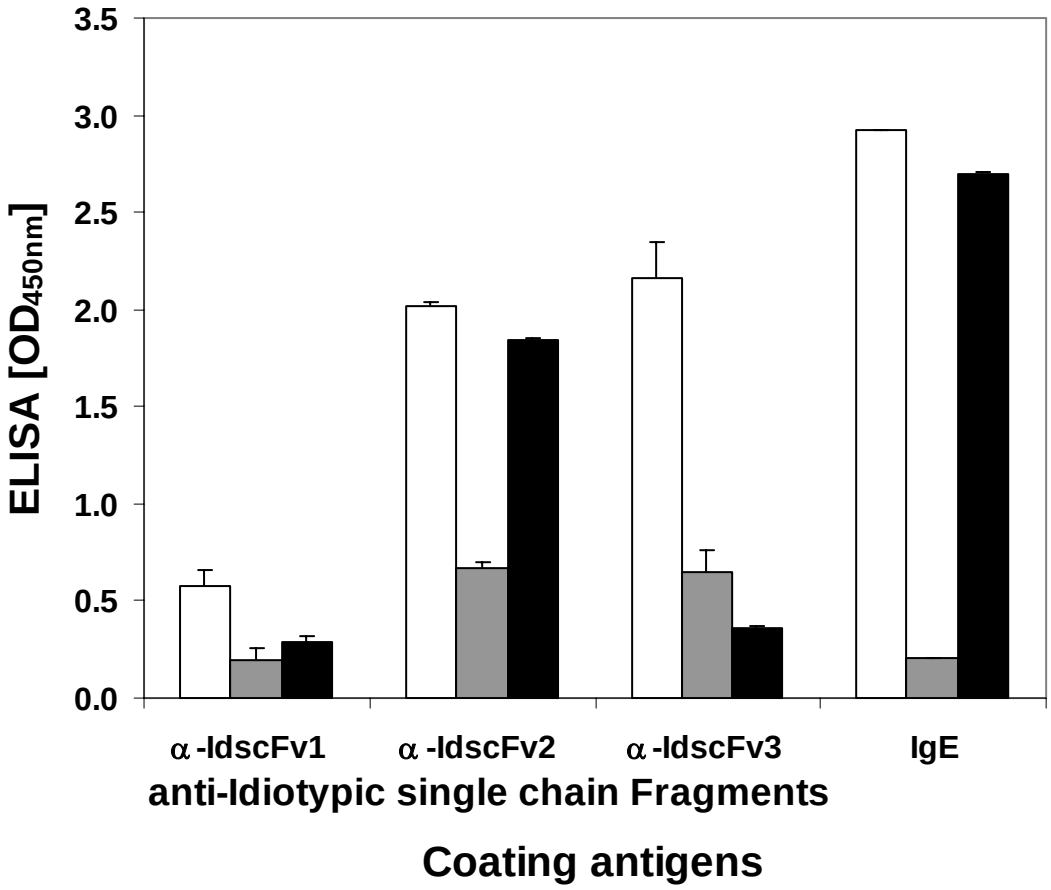


Fig. 2A,B

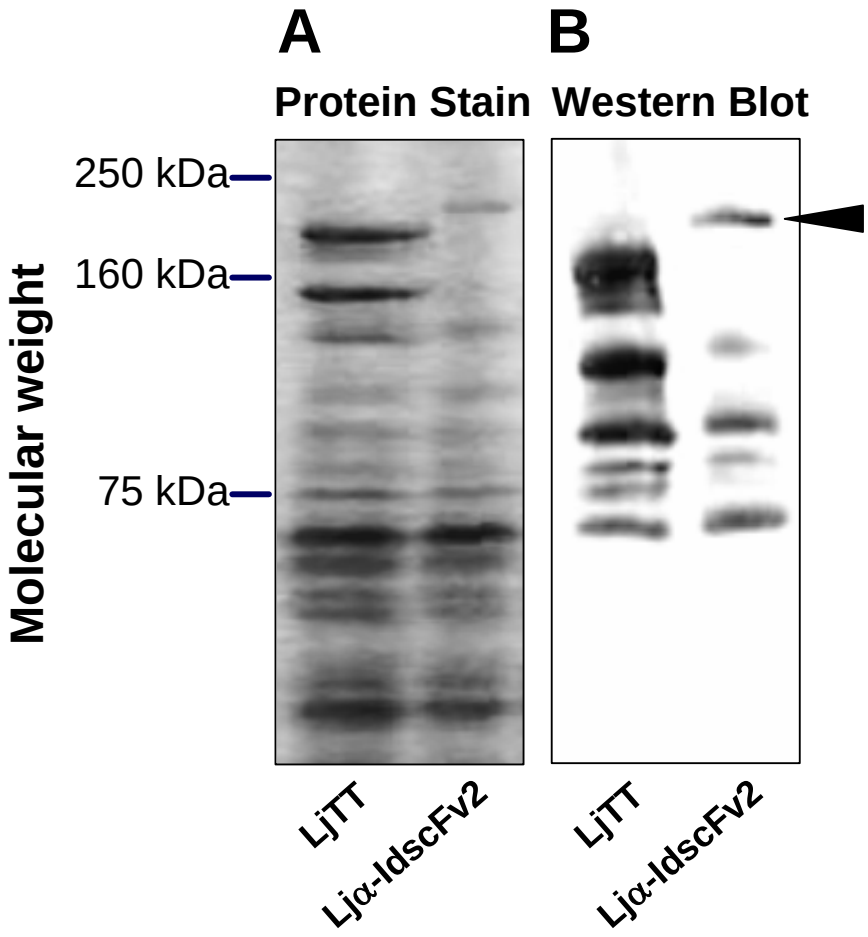

A
B
Protein Stain
Western Blot
250 kDa
160 kDa
75 kDa
Molecular weight
LjTT
Ljα-IdscFv2
LjTT
Ljα-IdscFv2

Fig. 2C

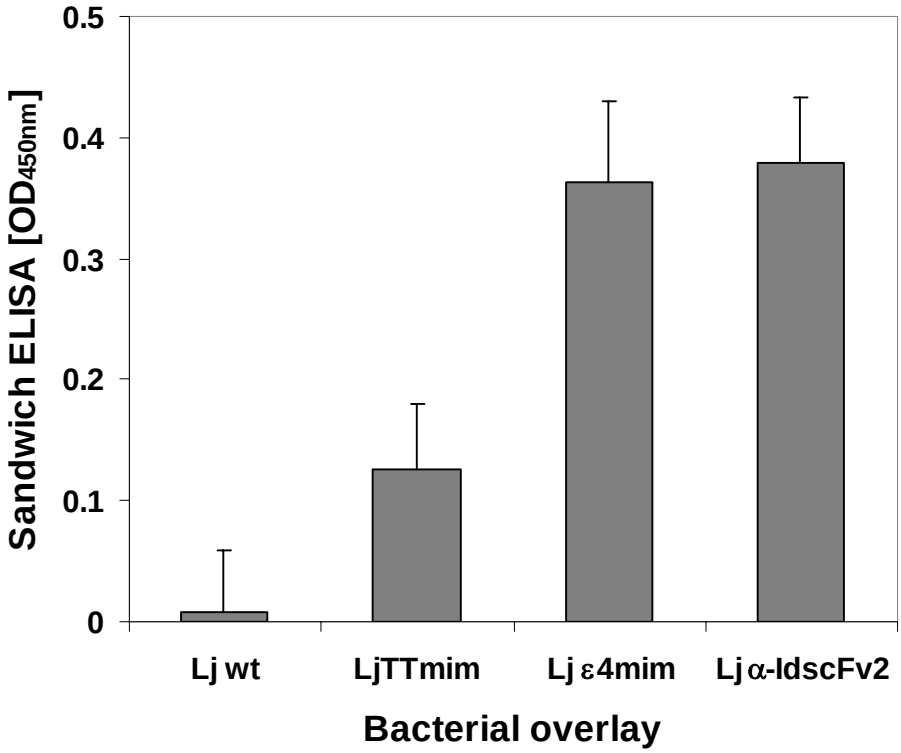
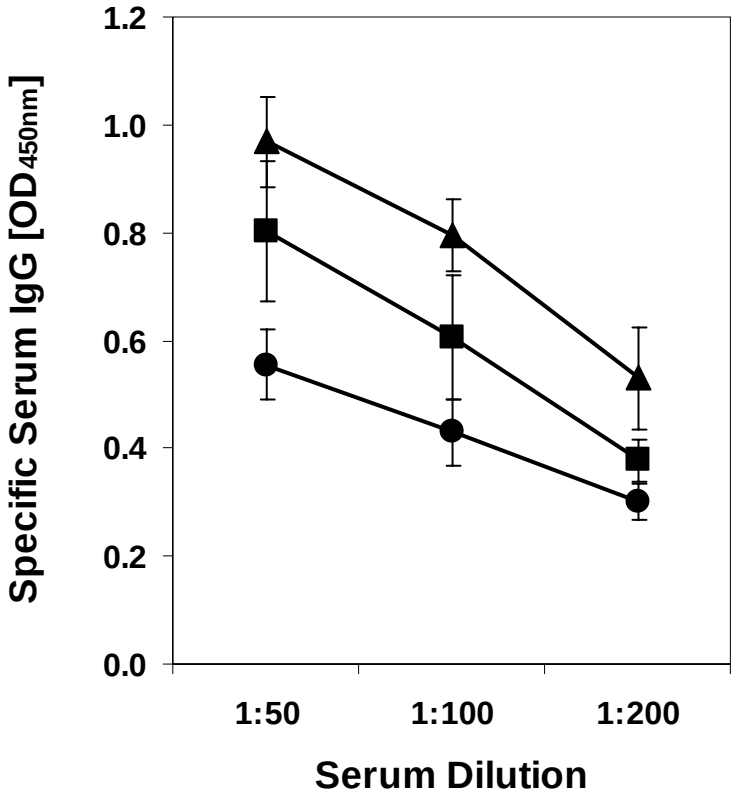

0.5
0.4
0.3
0.2
0.1
0
Sandwich ELISA [OD450nm]
Lj wt
LjTTmim
Lj ε4mim
Lj α-IdscFv2
Bacterial overlay

Fig. 3A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1.0
0.8
0.6
0.4
0.2
0.0
Specific Serum IgG [OD450nm]
1:50
1:100
1:200
Serum Dilution

Fig. 3B

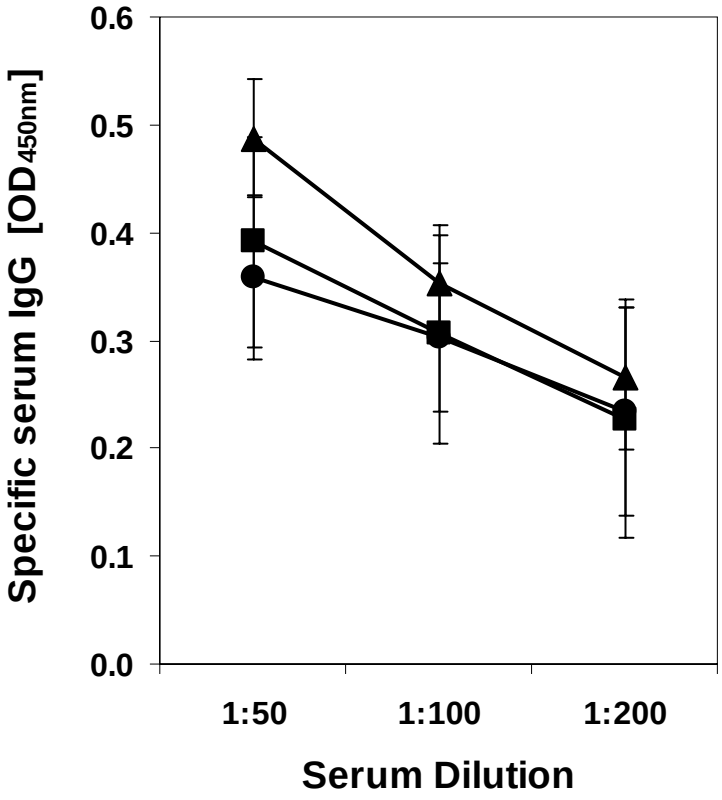
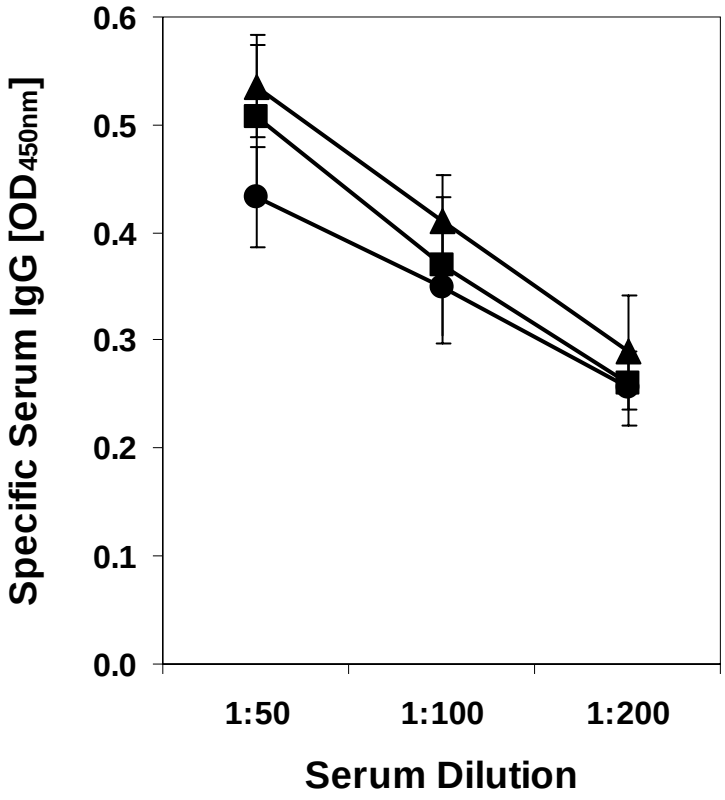

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Specific serum IgG [OD450nm]
1:50
1:100
1:200
Serum Dilution

Fig. 4A


Specific Serum IgG [OD450nm]
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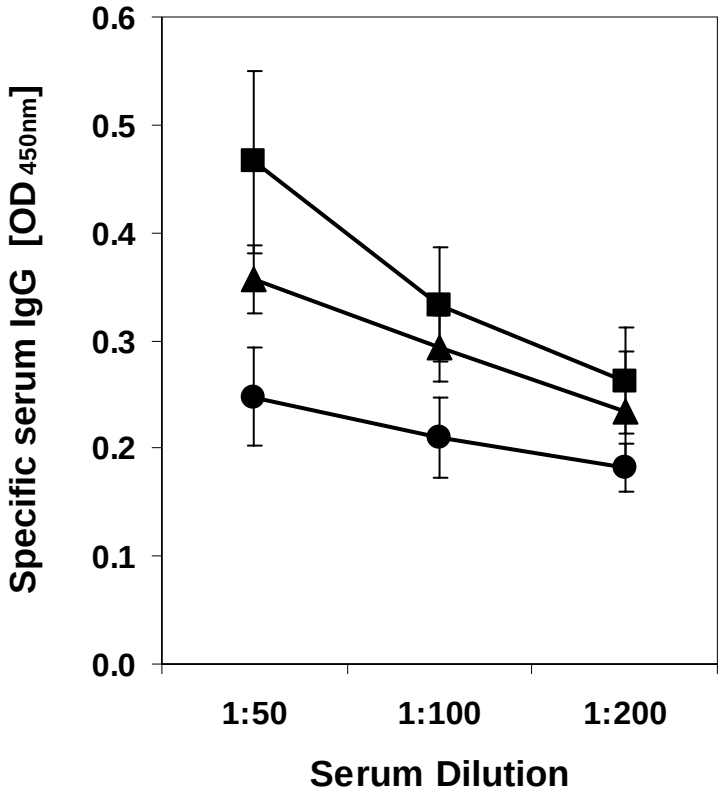
0.5
0.4
0.3
0.2
0.1
0.0
1:50
1:100
1:200
Serum Dilution

Fig. 4B


0.6
0.5
0.4
0.3
0.2
0.1
0.0
Specific serum IgG [OD 450nm]
1:50
1:100
1:200
Serum Dilution

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A2 Scheppler L., Zürcher A.W., Germond J.E., Vogel M., Stadler B.M. Oral Vaccination of mice against Tetanus with recombinant Lactic Acid Bacteria. SGAI/SSAI Meeting 2000. Basel.

A3 Scheppler L., Germond J.E., Vogel M., Miescher S.M., Stadler B.M. Surface display of a Single-Chain Fv Antibody in *Lactobacillus johnsonii* (La1) for Oral Vaccination. SGAI/SSAI Meeting 2001. Lausanne.

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A5 Scheppler L., Marti P., Vogel M., Germond J.E., Fritsché R., Miescher S.M., Stadler B.M. Expression of an anti-idiotypic Antibody on the surface of recombinant *Lactobacillus johnsonii*. SGAI/SSAI Meeting 2002. Lugano.

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